POPULATION GENETICS, SYSTEMATICS, BIOGEOGRAPHY, AND EVOLUTION OF THE SOUTHEASTERN CENTRAL TEXAS *EURYCEA* CLADE BLEPSIMOLGE (PLETHODONTIDAE)

by

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ABSTRACT

POPULATION GENETICS, SYSTEMATICS, BIOGEOGRAPHY, AND EVOLUTION OF THE SOUTHEASTERN CENTRAL TEXAS *EURYCEA* CLADE BLEPSIMOLGE (PLETHODONTIDAE)

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The central Texas *Eurycea* are a diverse group of perennibranchiate salamanders which inhabit the Edwards Plateau. Although several studies have addressed the systematics and taxonomy of this group, little is known about species boundaries within the southeastern Blepsimolge. Here I assess genetic variation and phylogenetic relationships both within and among species of the southeastern Blepsimolge. Using mitochondrial DNA sequences of the control region, I examine the population structure and genetic variation of *Eurycea nana* as well as the evolution of the control region within *Eurycea* and other salamanders. I compared levels of genetic variation among 24 *E. nana* from San Marcos Springs (Hays Co.), individuals from a

geographically proximate population at Comal Springs (Comal Co.), and representatives of several other central Texas *Eurycea*. No population structure was found among the three *E. nana* localities sampled and haplotype diversity in *E. nana* was lower than in *E. sp.* Comal Springs, which is consistent with previous results.

To examine species boundaries and evolutionary patterns among the southeastern Blepsimolge, I used three mitochondrial DNA products (control region, cytochrome *b*, and partial ND2), totaling 3,252 base pairs, to reconstruct phylogenetic relationships. The combined data set consisted of 58 ingroup populations or taxa from 43 different localities sampled from caves and springs throughout the eastern portion of the Edwards Plateau, south of the Colorado River. Several species boundaries recovered in this study do not appear to be wholly consistent with previous designations. *Eurycea latitans* is paraphyletic and appears to be conspecific with *E. tridentifera*. Populations of *Eurycea pterophila* form a weakly-supported monophyletic group and the range of this species extends beyond springs and caves in the Blanco River basin, contrary to previous inferences. Deep divergences within the southeastern Blepsimolge among taxa distributed along the Edwards fault zone aquifer suggest that formation of the Edwards Aquifer and compartmentalization within it may have been integral to the early diversification of this group. Subsequent dispersal and diversification probably ensued when the Lower Glen Rose limestone in the central portion of their distribution was exposed. Aquifer and cavern development in the region provided access to novel habitat that was exploited by salamanders. Finally, phylogenetic analysis revealed recurring patterns of morphological convergence among hypogean populations with cave-associated morphologies.

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CHAPTER 1

INTRODUCTION

Members of the plethodontid salamander genus *Eurycea* in central Texas occur as relictual, disjunct populations confined to springs, caves with water and other underground pools and streams (Sweet 1982; Sweet 1984; Chippindale 2000; Chippindale et al. 2000). Their distribution is confined to the Edwards Plateau, a region of uplifted Cretaceous limestone that has been eroded and dissected over time to form many caves and one of the most productive karst aquifers in the world. The geographic range of central Texas *Eurycea* includes the interior Edwards Plateau exclusive of the Llano uplift (Sweet 1982; Chippindale et al. 2000) and extends southwest along the Balcones Escarpment from Bell County to Bexar County and west to Val Verde County.

With very few exceptions, central Texas *Eurycea* are perennibranchiate, obligatory paedomorphs; that is, they do not metamorphose and reproductively mature adults retain gills and other larval features associated with a strictly aquatic life history. The evolution of paedomorphosis within this group is presumed to have been triggered by periods of drought during the Miocene. Xerification of the landscape during this epoch coincided with uplift of the Edwards Plateau (Sweet 1978a) and ancestral biphasic populations persisted only in relatively scarce mesic areas such as deep canyons and spring outflows. Under this scenario, the current taxonomic diversity observed in this clade is likely the result of allopatric speciation facilitated by a serendipitous combination

of climatic conditions, geologic change, and life history. Members of this clade are restricted to sites with permanent water and have relatively small geographic ranges and confinement to an aquatic life history in a xeric region may explain the considerable species diversity within this group, some of which has only recently been recognized (e.g., five recent species descriptions in: Chippindale et al. 1993; Chippindale et al. 2000; Hillis et al. 2001) while additional species are still awaiting description (Chippindale et al. 2000; Chippindale personal communication; Hillis personal communication).

The highly fragmented, restricted habitats of these salamanders, coupled with the fact that some occur in densely populated areas that depend heavily on the local aquifers, led to federal and state protection for several species. For example, *E. sosorum* (a federally endangered species) is known only from a handful of outflows in Barton Springs, located in Zilker Park, Austin, Texas (Chippindale et al. 1993). This species is endemic to a single small area, located in the heart of a rapidly growing metropolis and both the quality and quantity of the aquifer water discharged by Barton Springs are threatened by urbanization. *Eurycea nana* is another federally protected species endemic to spring outflows at the headwaters of the San Marcos River and a short stretch of river downstream of the springs (Tupa and Davis 1976; Nelson 1993). *Eurycea nana* and *E. sosorum* occupy similar microhabitats (gravel beds around spring outflows) and each occurs in sympatry with another troglobitic species: *E. rathbuni* in San Marcos Springs and *E. waterlooensis* in Barton Springs. This pattern of pairs of sympatric surface and subterranean species also occurs at Comal Springs, in Comal County. The status of both the surface and subterranean species at this site is problematic. The surface species

clearly is closely related to *E. sosorum, E. nana,* and other primarily spring-dwelling species in the area, while the aquifer-dweller is a member of the Typhlomolge clade sensu Hillis et al. (2001) (Chippindale et al. 2000; Chippindale, personal communication; unpublished data).

The central Texas *Eurycea* display a remarkable degree of morphological variation which tends to be habitat-associated. Epigean populations (those inhabiting surface waters) are found in springs, areas around spring outflows, and first order streams typically have an appearance similar to that of the larvae of other spelerpines (Spelerpinae is a subfamily of the family Plethodontidae and occurs in eastern North America; Chippindale et al. 2004). Hypogean populations (those inhabiting subterranean aquatic habitats), however, exhibit a range of morphological features. The most extreme examples of cave-associated morphologies are *E. rathbuni, E. robusta,* and *E. waterlooensis*. These species have skin that lacks nearly all dark pigmentation; reduced eyes; thin, elongate limbs; shortened trunks (relative to other Texas *Eurycea*); and elongated, flattened snouts (Potter and Sweet 1981). Additionally, the degree to and frequency of which these cave-associated morphological traits are expressed varies among hypogean *Eurycea* populations (reported in great detail by Sweet 1984). Morphological similarities among populations that occur in similar habitats (surface or subterranean) misled earlier taxonomists into grouping salamanders with comparable phenotypes, as well as separating those with dissimilar phenotypes (e.g. Wake 1966). For example, *E. rathbuni, E. robusta,* and (briefly) *E. tridentifera* were placed in the genus *Typhlomolge* by Wake (1966) and despite evidence presented by Potter and Sweet

(1981), their taxonomic placement was not fully-resolved until recently (Chippindale et al. 2000; but see also Mitchell and Reddell 1965 and Mitchell and Smith 1972). Molecular systematic techniques have been integral in helping scientists resolve relationships among the central Texas *Eurycea* (Chippindale et al. 2000; Chippindale 2000; Hillis et al. 2001).

The central Texas *Eurycea* are divided into several geographically and genetically distinct groups. Hillis et al. (2001), following PhyloCode, a formal set of rules governing phylogenetic nomenclature (de Quieroz and Cantino 2001), named five clades within the central Texas *Eurycea*: Paedomolge (the clade containing all central Texas *Eurycea)*; Septentriomolge (*Eurycea tonkawae, E. naufragia,* and *E. chisolmensis)*; its sister group, Notiomolge, which contains two sister clades: Typhlomolge (*E. rathbuni*, *E. robusta,* and *E. waterlooensis)* and Blepsimolge (*E. latitans, E. pterophila, E. nana, E. neotenes, E. tridentifera, E. troglodytes, and E. sosorum).* All Septentriomolge occur north of the Colorado River along the Edwards Plateau fault zone (also known as the Balcones Escarpment). Typhlomolge inhabit underground water systems south of the Colorado River along the Balcones Escarpment where they are sympatric with two species of Blepsimolge (*E. nana* and *E. sosorum*). Blepsimolge are distributed throughout the Edwards Plateau as far west as Val Verde Co. and can be divided into two genetically and geographically distinct groups: the southwestern and southeastern Blepsimolge.

In the following chapters, I present an examination of population genetics, systematics, biogeography, and evolution of the most species-rich of the aforementioned groups, the southeastern Blepsimolge. The goals of the next chapter are three-fold: to

address the genetic diversity and genetic structure of *E. nana* and compare it to the nearby Comal Springs population; to examine the usefulness of the mitochondrial control region both as a marker for population genetic and phylogenetic studies of this group; and to determine the structure of the mitochondrial control region in comparison to other salamanders and other vertebrates.

The third chapter of this thesis focuses on a further investigation of the relationships among populations and species of the southeastern Blepsimolge using sequence data from the mitochondrial control region and two other mitochondrial genes. I assess the current taxonomy and systematics of this group and propose a new biogeographic hypothesis to explain their evolution and distribution. Finally I discuss the evolution of cave-associated features and address several possible mechanisms to explain patterns of morphological convergence within this group.

CHAPTER 2

APPLICATION OF MITOCHONDRIAL CONTROL REGION FOR POPULATION GENETIC AND PHYLOGENETIC STUDIES IN CENTRAL TEXAS *EURYCEA* AND ITS EVOLUTION IN SALAMANDERS

2.1 Introduction

The mitochondrial (mt) control region (CR), also known as the displacement loop (D-loop), is the largest non-coding portion of the mt genome, and contains several regions associated with "control" of replication and transcription (e.g., Clayton 1991). Several empirical studies have addressed the function of conserved regions, such as a site associated with the termination of replication (TAS; Doda et al. 1981; Cairns and Bogenhagen 1986) and conserved sequence blocks (CBSs) proposed as regulatory signals for the processing of the RNA primers for H-strand replication (Walberg and Clayton 1981). Comparative studies using sequence data have reported additional conserved regions (Mignotte et al. 1987; Foran et al. 1988; Quinn and Wilson 1993; Sbisá et al. 1997; Randi and Lucchini 1998; Ray and Densmore 2002). These regions have been proposed to have functional importance based on conservation across a broad range of taxa including birds (Quinn and Wilson 1993), mammals (Sbisá et al. 1997), and crocodilians (Ray and Densmore 2002). With the exception of *Xenopus* (Cairns and Bogenhagen 1986), few studies have examined the structure of the control region in amphibians in general or salamanders in particular (but see McKnight and Schaffer 1997).

In addition to conserved regions of functional importance, the CR also contains domains which evolve extremely rapidly. The CR is a particularly useful marker for genetic studies partly due to the rapid evolution of these non-conserved regions. Repeat units within the control region as well as an adjacent intergenic spacer have been reported in a variety of taxa, potentially making them useful for population level analyses. These repeats can be highly variable even within individuals (e.g. Brown et al. 1996); however heteroplasmic repeats can pose difficult problems to address in molecular studies (for review, see Lunt et al. 1998).

My interest in the control region is two-fold: its evolution in salamanders in comparison with other vertebrate taxa, and its utility as a fast-evolving marker for assessing genetic variation in central Texas spring and cave salamanders (*Eurycea*). The control region has been used in various studies which examine population structure and genetic variation in endangered or highly endemic species (e.g. Smith et al., 2002; Brogdon et al. 2003; Johnson et al. 2003; Lecis and Norris 2004; Oshida et al. 2004). Many salamanders of the genus *Eurycea* in central Texas have extremely restricted ranges (Chippindale et al. 1993; Chippindale et al. 2000; Hillis et al. 2001), and several are protected by federal or state mandate (Table 2.1). The San Marcos Salamander (*Eurycea nana*) is endemic to a single locality. In a previous study of allozyme variation in central Texas *Eurycea*, heterozygosity was found to be very low for *E. nana* (1.7%), compared to some other species endemic to similar regions of spring outflow (Chippindale et al. 2000). The restricted range and low molecular genetic variation in *E. nana* make it a suitable subject to examine the utility of the control region variation as a

molecular marker for studies of central Texas *Eurycea* because numerous species have a much larger range than *E. nana*, and those with similar restricted ranges have been reported to have higher allozyme heterozygosity (Chippindale et al. 2000). Thus, if the control region is an informative marker for a population genetic analysis of *E. nana* it is likely to be useful in other central Texas *Eurycea* species as well.

Eurycea nana was described from outlets of San Marcos Springs at the headwaters of the San Marcos River, Hays Co., Texas (Bishop 1941). The range of *E. nana* extends from the headwaters of the San Marcos River at Spring Lake to a stretch of river downstream (up to 150m) of the Spring Lake dam in San Marcos, Texas (Tupa and Davis 1976; Nelson 1993). Threats to its survival include human disturbances that result in reduced aquifer flow to the springs, increased silt accumulation, and pollutants, as well as introduced predator or competitor species. With its limited range and vulnerability to disturbances, this species is highly susceptible to decreases in population size or extinction. Because small populations are more likely to inbreed and are more susceptible to the effects of genetic drift, they are prone to reduced genetic variability which can lower the overall fitness of the individuals (Storfer 1999). Bottlenecking can increase the genetic load of the population (Lynch et al. 1995) as well as decrease overall heterozygosity (e.g. Bellinger et al. 2003; Johnson et al. 2004). These processes can lead to the extinction of small populations (Meffe and Carroll 1997). Therefore it is important to document genetic diversity of threatened species which creates a baseline of information to ensure that proper conservation decisions can be made.

Despite the apparent homogeneity of its habitat and therefore high probability of unrestricted gene flow within this species, I identify two possibilities for development of genetic structure within *E. nana*. *Eurycea nana* is occasionally found a short distance downstream of a man-made dam in Spring Lake, which may impede gene flow. Additionally, dye-trace studies have shown that the springs which feed Spring Lake originate from different sources (Ogden et al. 1986) and thus may connect to unknown populations of *E. nana* or other populations of central Texas *Eurycea*. If *E. nana* interbreeds with other *Eurycea* through these spring passages, this could compromise the genetic integrity of the population by impairing the rate of adaptive evolution, obscuring species boundaries, and altering mating curves. Although *E. nana* appear to be morphologically distinct from other Texas *Eurycea* (Chippindale et al. 1998), occasional introgression (at distant unknown and/or subterranean localities) may not be apparent in the population at San Marcos Springs if only considering phenotypic variation. In such a case, a survey of mitochondrial haplotype variation could reveal gene flow.

In addition to the suitability of the control region marker for population genetics, I also address its ability to resolve species level relationships within the central Texas *Eurycea*. Previous investigations of central Texas *Eurycea* have documented low genetic divergence among taxa using cytochrome b sequences (Chippindale et al. 2000; Hillis et al. 2001) and allozyme frequency data (Chippindale et al. 2000) and thus, several key taxonomic and phylogenetic problems remain unresolved.

Here I present a comprehensive study of control region variation in the central Texas *Eurycea*. Analyzing samples from the Federally Threatened *E. nana*, I increase

the available information about the genetic variation of this species and test for population structure. Additionally, I offer a comparison with a nearby *Eurycea* population with unresolved taxonomic status at Comal Springs, previously assigned to *E. nana* (but see Chippindale et al. 1998) and reconstruct the phylogeny of other closely related Texas *Eurycea*. I also extend my analysis to include a comparison of the salamander control region to other vertebrate taxa. Conserved features of the control region from several vertebrate groups are compared in six salamander families to identify regions of known functional importance and other potentially important conserved regions.

2.2 Materials and Methods

2.2.1 Sample Collection and Laboratory Methods

A total of 24 specimens of *E. nana* were collected from three sites at San Marcos Springs, Hays County, Texas: a large aquifer outflow (Diversion Spring) near the center of Spring Lake, a site near the hotel (at the north end of the lake), and a site below the dam, several meters downstream of Spring Lake between 1988 and 2004 (Figure 2.1). Nine specimens were collected between 1990 and 2005 from several spring orifices (no more than 30 m apart) at Comal Springs, the headwaters of the Comal River in Comal County, Texas. Accession numbers for museum specimens (where available) are listed in Appendix A.

DNA was extracted from muscle or liver tissue using DNeasy DNA extraction kits (Qiagen). The mitochondrial control region was amplified and directly sequenced with a series of primers (Table 2.2) flanking the control region in the adjacent

cytochrome b and 12S rRNA genes. PCR products were amplified using Hot Start *ExTaq* (Takara-Mirus) on MJ Research PTC thermal cyclers using primers flanking the control region and adjacent tRNA genes. PCR conditions which yielded the most consistent results were as follows: Reactions consisted of 1-2 µl of dilute DNA (typically 10-50 ng of DNA), 0.5-1.0 µM of each primer, 0.75 mM dNTPs mix, polymerase buffer (1.5 mM $MgCL_2$), and 1-2 U *ExTaq* polymerase in a total volume of 20 µl. Occasionally, 0.5 µl of 100% DMSO was used in PCR reactions if amplification was difficult. Thermal cycling conditions varied greatly depending on the template of interest and difficulty of amplification. Typical conditions are as follows: Step 1: 96° 3 min; Step 2: Annealing temp. 50° 30 s.; Step 3: 72° 1min/kb; Step 4: 96° 20 s; Step 5: repeat steps 2-4 (x 30); Step 6: 72° 10 min; Step 7: 4° hold. Single stranded DNA and free dNTPs were removed from PCR products using a combination of exonuclease I and shrimp alkaline phosphatase enzymes (USB). Both strands of each PCR product were sequenced for moderate to complete overlap (overlap in heteroplasmic repeats not possible) using ABI Big Dye v3.1 chemistry. Unincorporated products were precipitated with ethanol containing 0.75M sodium acetate and 125mM EDTA. Applied Biosystems 377 and 3130xl automated sequencers were used to obtain the sequence data.

2.2.2 Data Analysis

Raw sequence chromatograms were edited with Sequencher 4.2 (Gene Codes Corp.). The ClustalW algorithm implemented in MacVector 7.2.3 (Accelrys) was used to perform multiple alignments of nucleotide sequences. Three separate alignments were constructed as follows: (1) All sequences of *E. nana* for population genetic analyses; (2)

sequences of *E.* sp. Comal Springs for population genetic analyses; (3) an alignment including representatives of all members of the southeastern Edwards Plateau Blepsimolge clade (Hillis et al. 2001) including all samples of *E. nana* and *E.* sp. Comal Springs for phylogenetic analysis. Gaps and insertions/deletions (indels) were adjusted manually to account for indels associated with long base-pair runs and to maximize inferred sequence homology. DnaSP v4.10.4 (Rozas et al. 2003) was used to calculate nucleotide diversity (π, Nei and Tajima 1983) and haplotype diversity, *h* (Nei and Tajima 1981). Analysis of molecular variance (AMOVA) was performed using Arlequin (Schneider et al. 2000) to test for genetic structure among the three sampled sites for *E. nana*. Alignment gaps were considered to be informative in the calculation of haplotype frequencies for the AMOVA analysis. I also reconstructed a phylogenetic hypothesis for the southeastern Blepsimolge clade (including all populations in this study) using *E.* sp. Pedernales (Chippindale et al. 2000) as the outgroup. Maximum parsimony analysis was carried out in TNT (Goloboff et al. 2003b) using the "traditional search," TBR branch swapping and 10 random-taxon-addition replicates. Support for each node was evaluated by nonparametric bootstraping with 1000 psuedoreplications, each with 10 random-taxon-addition replicates. MrBayes v3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used to reconstruct a Bayesian-based phylogeny with posterior probability support values. I applied the evolutionary model selected by Modeltest v3.7 (Posada and Crandall 1998) under the Akaike Information Criterion (AIC) for two data partitions, intergenic spacer (IGS) plus $tRNA^{PRO}$ and CR alone. For the phylogenetic analyses, repeat units within the IGS were excluded from all analyses because of

heteroplasmy and difficulty in assessing homology, and gaps/indels were treated as missing data.

2.2.3 Mapping of Conserved Motifs to the Salamander D-loop

Control region sequences from *E. nana* and other salamanders retrieved from GenBank (*Andrias davidianus* NC004926; *Ambystoma laterale* NC006330; *Ranodon sibiricus* NC004021; *Lyciasalamandra atifi* NC002756; *Rhyacotriton variegates* NC006331; *Hemidactylium scutatum* NC006342) were used for comparison of conserved regions to other vertebrate taxa. The TAS was mapped in comparison to empirically delimited TAS in *Xenopus* (Cairns and Bogenhagen 1986). The identification of three control region domains and the conserved sequence blocks (CSBs) were determined by comparison to those in mammals (Sbisá et al. 1997).

2.3 Results and Discussion

2.3.1 Control Region Features and Evolution

2.3.1.1 Intergenic Spacer

The control region is flanked on the 5' end of the heavy strand by $tRNA^{PRO}$ and an intergenic spacer (IGS; Figure 2.2), which has also been observed in ambystomatid salamanders (McKnight and Shaffer 1997) and other plethodontids (Mueller and Boore 2005). Direct sequencing of intergenic spacer PCR products and of clones from *E. nana* and *E.* sp. Comal Springs revealed that numerous IGS haplotypes were present within individuals. The IGS contains two different variable number tandem repeats (VNTRs), which I refer to as type I and type II. Type I repeats vary in length from 45 to 50 bp and contain a poly-T region (five to eight bp) within each repeat. Type II repeats are shorter

in length (11 bp) and exhibit less variation than type I repeats, both in sequence and copy number. Sequencing through the type I repeats within the IGS was often difficult. Primers were used to sequence from both directions up to the VNTR region, but due to the presence of multiple IGS species in some individuals, accurate reads were sometimes impossible. Repeats were removed from the alignments because of difficultly sequencing as well as difficulty in establishing homology among repeats. However, the last type I repeat and type II repeats were included in the alignments used for the AMOVA and phylogenetic analyses. These repeats were easily sequenced due to their position, and both are only partial repeats which appear to be conserved among all *Eurycea* included in this study.

Heteroplasmy was primarily associated with length variation of type I repeats and variation in number of type I repeats; however a few individuals exhibited variation in number of type II repeats (based on observation of an "out-of-phase" sequence electropherogram pattern). Seven of nine Comal Springs individuals and 17 of 24 individuals of *E. nana* appeared to be heteroplasmic.

Heteroplasmic VNTRs in the control region or IGS are not uncommon and have been reported in a range of organisms (Lunt et al. 1998) including salamanders (Mueller and Boore 2005). The use of heteroplasmic VNTRs to address questions in molecular ecology has been criticized, owing to the difficulty of correctly assessing allele frequencies, variation among different tissues, and uncertainty of the mechanisms that generate mtVNTR diversity (Lunt et al. 1998). These are valid concerns and in my data set it was impossible to quantify intra-individual VNTR. A possible mechanism for the

source of length heteroplasmy involves frequent competitive misalignment within the repeat region prior to replication (Buroker et al. 1990). This model requires a TAS imbedded within the repeats, and repeats within the IGS of *Eurycea* do not appear to have the TAS associated with them.

2.3.1.2 Control Region

VNTRs were not present in the control region of *Eurycea* or other species in this study. Several conserved sequences observed in other vertebrates have been identified by sequence similarity (Figure 2.2). Three domains of the D-loop are currently recognized in vertebrates: the extended termination associated sequence (ETAS) domain I, the central conserved domain II, and the CSB domain III (Sbisá et al. 1997). Domain I is adjacent to $tRNA^{PRO}$ and contains one or two copies of the ETAS, identified as conserved regions containing TAS sequences in mammals (Sbisá et al. 1997), birds (Randi and Luchinni 1998), and crocodiles (Ray and Densmore 2002). Here I identified the TAS in salamanders by comparison to the experimentally determined TAS in *Xenopus* (Cairns and Bogenhagen 1986) and its consensus in salamanders studied is ATTATGYATATCGTA. The TAS occurs at variable distances from $tRNA^{PRO}$, and the region preceding the TAS is highly variable. The TAS occurs within a conserved region (ETAS), which aligns to the TAS reported by McKnight and Shaffer (1997). However I did not identify a second ETAS as found in mammals (Sbisá et al. 1997). Sbisá et al. (1997) suggested a possible functional role for ETAS2, although its absence in salamanders suggests that this function may not be conserved across vertebrates.

Domain II occurs in between Domains I (ETAS domain) and III (CSB domain), and generally is conserved. The function of this region is not known, although several conserved blocks show high sequence similarity to the mammalian and crocodilian conserved central domain (Figure 2.3). What appears to be conservation of this region across tetrapods is an indication of its importance.

Domain III, the CSB domain, is highly variable among salamanders with the exception of the CSBs. CSB2 and CSB3 appear to be relatively conserved among salamanders, while CSB1 is conspicuously truncated (Figure 2.4). This shortened CSB1 version was reported in *Xenopus* (Cairns and Bogenhagen 1986) and may be common to all amphibians. In mammals and birds CSB1 appears to be the only conserved CSB (Sbisá et al. 1997; Randi and Luchinni 1998) and has been suggested as the only functionally essential CSB (Sbisá et al. 1997). However a shortened CSB1 has also been reported in crocodilians and thus its function in vertebrates may be related to the presence of the sequence RCATA (Ray and Densmore 2002). Amphibians may have an alternate CSB whose function is identical to that of CSB1 in other vertebrates.

2.3.2 Intraspecific Variation

The total length of the alignment for all *Eurycea* in this study was 1237 bp. Alignments for only *E. nana* and only *E.* sp. Comal Springs were 1210 and 1201 bp respectively. This does not include the length heteroplasmy of repeats within IGS; repeats were removed from alignments due to difficulty in establishing homology and acquiring clean sequence data from this region. With gaps included, seven haplotypes were observed among 24 individuals of *E. nana* and five were observed among nine

individuals of *E.* sp. Comal Springs (Table 2.3). All other *Eurycea* had unique haplotypes. The most common haplotype within *E. nana*, hap1, was present in 17 individuals, followed by hap3 (two individuals), and the remaining were unique haplotypes. Seventeen variable sites were observed in the *E. nana* alignment (six of them were parsimony informative; pi), while five variable sites were observed for the Comal Springs population (one pi). All unique *E. nana* haplotypes were distinguished by multiple substitutions and/or indels. Among the other Blepsimolge, all had unique haplotypes with the exception of *E. tridentifera* and *E. latitans,* which share the same haplotype when not considering indels, although were different if indels were considered.

Comparing *E. nana* and *E.* sp. Comal Springs, nucleotide diversity estimates are within one standard deviation of each other and are low (Table 2.3). In contrast, haplotype diversity was estimated to be larger in *E.* sp. Comal Springs than in *E. nana* for both estimates of haplotypes diversity (Table 2.3). This result is consistent with that of Chippindale et al. (2000) who found higher allozyme heterozygosity in *E.* sp. Comal Springs (5.7%) than in *E. nana* (1.7%). This may be a reflection of potential differences in habitat stability (and thus fluctuations in population size) at Comal Springs compared to San Marcos Springs. This is a somewhat surprising result given that Comal Springs ceased to flow during the 1950's (the Drought of Record) and again in 1984 (Ogden et al. 1986). San Marcos Springs continued to flow during both of these drought periods (Ogden et al. 1986), suggesting it may be a more stable habitat for spring-dwelling species than Comal Springs. If *E. nana* is more resistant to population bottlenecks because of their stable spring habitat, then I would expect to observe higher genetic

diversity in populations at San Marcos Springs relative to populations at Comal Springs. However the phylogenetic tree suggests more recent gene flow among Comal Springs and *E. neotenes*, whereas *E. nana* appears to have been isolated longer. Additionally if *E.* sp. Comal Springs is able to migrate to subterranean habitat, this may negate the effects of reduced water flow to Comal Springs. Despite several possible explanations, this discrepancy is worth further examination given its possible implications for management of other threatened Texas spring endemics and considering the apparent similarity in habitats of these species. Both *E. nana* and *E.* sp. Comal Springs are restricted to single areas of high discharge along the Balcones Escarpment, which are host to other endangered species such as fishes (e.g., *Gambusia georgei* and *Etheostoma fonticola*) and invertebrates (e.g. *Stygoparnus comalensis, Stygobromus pecki* and *Heterelmis comalensis*).

It is important to note that my haplotype diversity estimates are reduced for both species due to the exclusion of the repeat region, which is highly variable. The type I repeat region is the most variable region of the IGS/control region even among apparently homoplasmic individuals.

The AMOVA did not detect population structure within *E. nana* (P = 0.96; Table 2.4). This is not a surprising result given the apparent homogeneity of their habitat, consisting of a fairly large and consistent upwelling of water from the Edwards aquifer into Spring Lake. However, sequence divergence among some haplotypes is relatively high within *E. nana* (as high as 0.7%), compared to that among three other species (0.4% between *E. pterophila* and *E. latitans* + *E. tridentifera*). High divergence within *E. nana*

may indicate that the springs that feed it are connected to other more distant, unknown populations (either conspecific or not), and there is occasional migration among these sites. This could explain the high divergence among *E. nana* haplotypes and the relatively low frequency of the most divergent haplotypes. An alternative explanation could be that *E. nana* at San Marcos contain divergent haplotypes because of persistent ancestral polymorphisms. I am currently developing microsatellite markers for a closely related species, *E. sosorum*, which may be applicable to population genetic studies of *E. nana*. This will enable me to determine whether nuclear markers give the same pattern as the mitochondrial ones and further explore patterns of genetic diversity.

2.3.3 Interspecific Variation

Parsimony and Bayesian analyses recovered similar trees, differing only in that the Bayesian analysis was better resolved than the parsimony analysis. Previous confusion in the literature regarding the distribution of *E. nana* led Chippindale et al., (1998) to clarify the status of *E. nana* as distinct from a population at Comal Springs (approximately 29 km southwest of San Marcos), which currently is treated as an undescribed species (Chippindale et al. 2000; Chippindale 2000). Taxonomic discrimination among these two populations (*E. nana* at San Marcos Springs and *E.* sp. at Comal Springs) was based on numerous unique, apparently fixed allozyme alleles within *E. nana*, two unique cytochrome *b* substitutions within *E. nana*, and several morphological and morphometric differences (Chippindale et al. 1998). My results support the recognition of these populations as independent lineages. Sequence divergence is approximately 3% (uncorrected p) between these two species, and *E.* sp.

Comal Springs is more closely related to *E. neotenes,* while *E. nana* forms a polytomy with *E. sosorum* and the clade of *E. neotenes, E. pterophila, E. tridentifera,* and *E. latitans* in the Bayesian and parsimony analyses (Figure 2.5). Sequence divergence was low among *E. tridentifera*, *E. latitans* and *E. pterophila* (0-0.4%; Figure 2.5), especially compared to within species variation of *E. nana* (0.7%). As mentioned above, this may indicate relatively divergent haplotypes within the gene pool of *E. nana* (suggestive of old ancestral polymorphism or gene flow with other undiscovered population[s]). However, such low divergence among species could also indicate hybridization (e.g. Sweet 1984), rapid speciation, or incomplete lineage sorting.

2.4 Conclusions

In addition to highly variable sites and length variation of VNTRs in the IGS, the CR has several regions which appear to be highly conserved among tetrapods. Because the CR and IGS also contain very fast evolving segments, this may be a valuable marker for assessing species boundaries within central Texas *Eurycea* and other amphibians. While numerous species had been described in the recent past, partly due to advances in molecular systematic techniques (Chippindale et al. 1993; Chippindale et al. 2000; Hillis et al. 2001), genetic diversity and species boundaries within this group of salamanders are poorly understood, particularly with respect to two putatively undescribed species (*E.* sp. Comal Springs and *E.* sp. Pedernales) and the *E. latitans* and *E. troglodytes* species complexes (Chippindale et al. 2000).

Species with restricted ranges that have become fragmented or have experienced severe population declines are more likely to be vulnerable to the effects of genetic drift

and loss of heterozygosity than more widespread species (e.g., Johnson et al. 2004). Knowledge of the genetic structure and associated parameters of these populations can be vital to their management and conservation. This is especially crucial for *E. nana*, with its extremely restricted range and threats to its habitat. This study provides a baseline of genetic diversity information, as well a comparison to other *Eurycea* with restricted ranges or of special concern. These results do not support the recognition of unique units of genetic diversity within *E. nana* for management purposes or otherwise. However, the contrast in haplotype diversity between *E. nana* and *E.* sp. Comal Springs populations, and origin of divergent haplotypes within *E. nana* warrant further investigation of these highly endemic salamanders.

2.5 Tables

Table 2.1. Federal and state protected and candidate species of Texas *Eurycea*. *Indicates same federal and state status.

Species	Protection	Status
Eurycea chisolmensis	F	Candidate
E. latitans	S	Threatened
E. nana	F/S	Threatened*
E. naufragia	F	Candidate
E. rathbuni	F/S	Endangered*
E. robusta	S	Threatened
E. sosorum	F/S	Endangered*
E. tridentifera	S	Threatened
E. waterlooensis	F	Candidate

Table 2.2. List of primers used during this study.

Primer Name	Sequence	Position	Direction
Blep12SDL1r	5'-GTGGCTGGCACGAGATTTAC-3'	12SrRNA	reverse
Blep12SDL2r	5'-GGTGGCTGGCACGAGATT-3'	12SrRNA	reverse
BlepCBDL1f	5'-CATYGCCGACACACTAGYACTTAC-3'	Cyt b	forward
EurCB5	5'-CCMTTYATTGAAATTGGACAAG-3'	Cyt b	forward
EurDL11	5'-CTCGCTTGAAAAACGTGTTGTGC-3'	Dloop	reverse
EurDL2r/c	5'-ATGCATATGTTAKTTCCAGGG-3'	Dloop	forward
EurDL3	5'-GTTAGTCCATAGATTCAAACCAG-3'	Dloop	reverse
EurDL5r/c	5'-CACTACTGACACCCAAAGC-3'	Dloop	forward

Table 2.3. Measures of genetic diversity in *E. nana* and *E.* sp. Comal Springs. Haplotype diversity = *h*; Nucleotide diversity = π. Standard deviations are given in brackets.

	Sample size	Alignment length (excluding gaps)	Number of haplotypes, no gaps	*h*, gaps considered	Number of haplotypes, with gaps	*h*, gaps excluded	π
Eurycea nana	24	1210 (1205)	7	0.504 [0.123]	6	0.380 [0.125]	0.0017 [0.0005]
E. sp. Comal Springs	9	1201 (1199)	5	0.833 [0.098]	3	0.639 [0.016]	0.0012 [0.0005]

Table 2.4. Summary of analysis of molecular variance in *Eurycea nana* from three sites in San Marcos. The source of variation is wholly attributed to within population variation and there is no among population variation. Levels of significance are based on 1000 random permutations.

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation	Fixation index: Fst	P
Among populations	2	13.483	-0.63904 Va	-5.62		
Within populations	22	264.157	12.00714 Vb	105.62	-0.05621	>0.95

2.6 Figures

Figure 2.1. Arial photograph of Spring Lake in San Marcos, Texas. Arrows indicate approximate sampling localities for *Eurycea nana*. A= Hotel site; B= Diversion Spring site; C= Below Dam site.

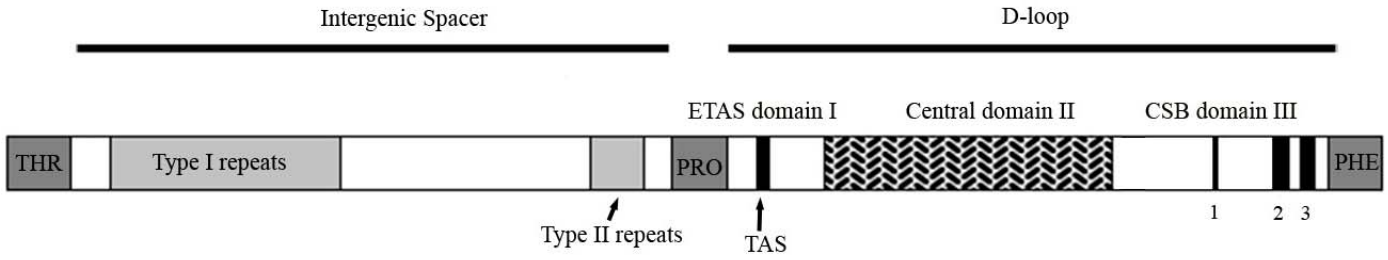

Figure 2.2. Features of the *Eurycea* control region (D-loop) and intergenic spacer. TAS- termination associated sequence; CSB- conserved sequence block; THR- Threonine tRNA; PRO- Proline tRNA; PHE- Phenylalanine tRNA.

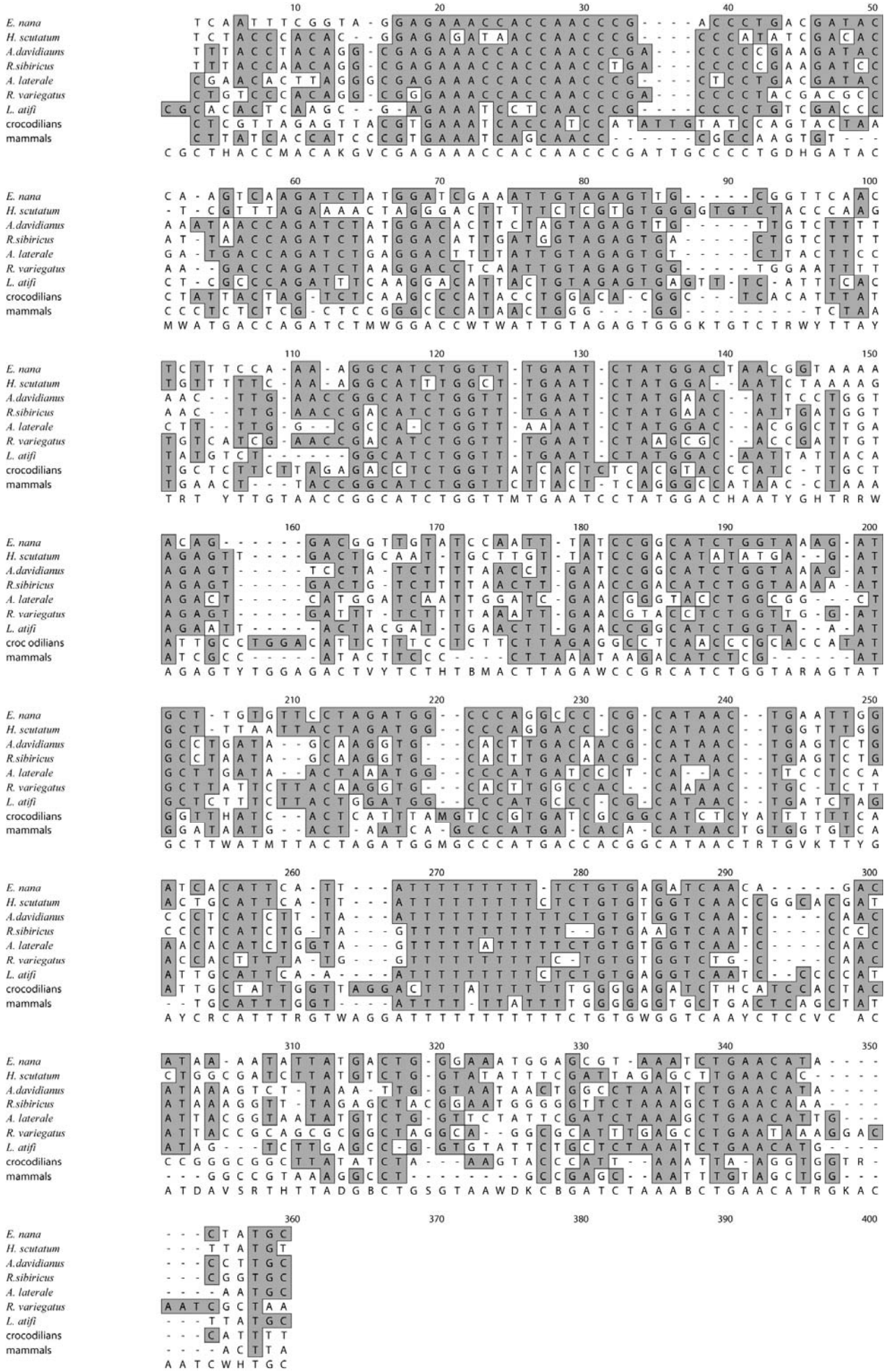

Figure 2.3. Central conserved domain. Representatives of six salamander families are compared to the mammalian (Sbisá et al. 1997) and crocodilian (Ray and Densmore 2002) consensus sequences. Shaded regions represent sequence similarities, with the consensus sequence on the bottom line. Extent of the central domain was determined by comparison to the mammalian consensus.

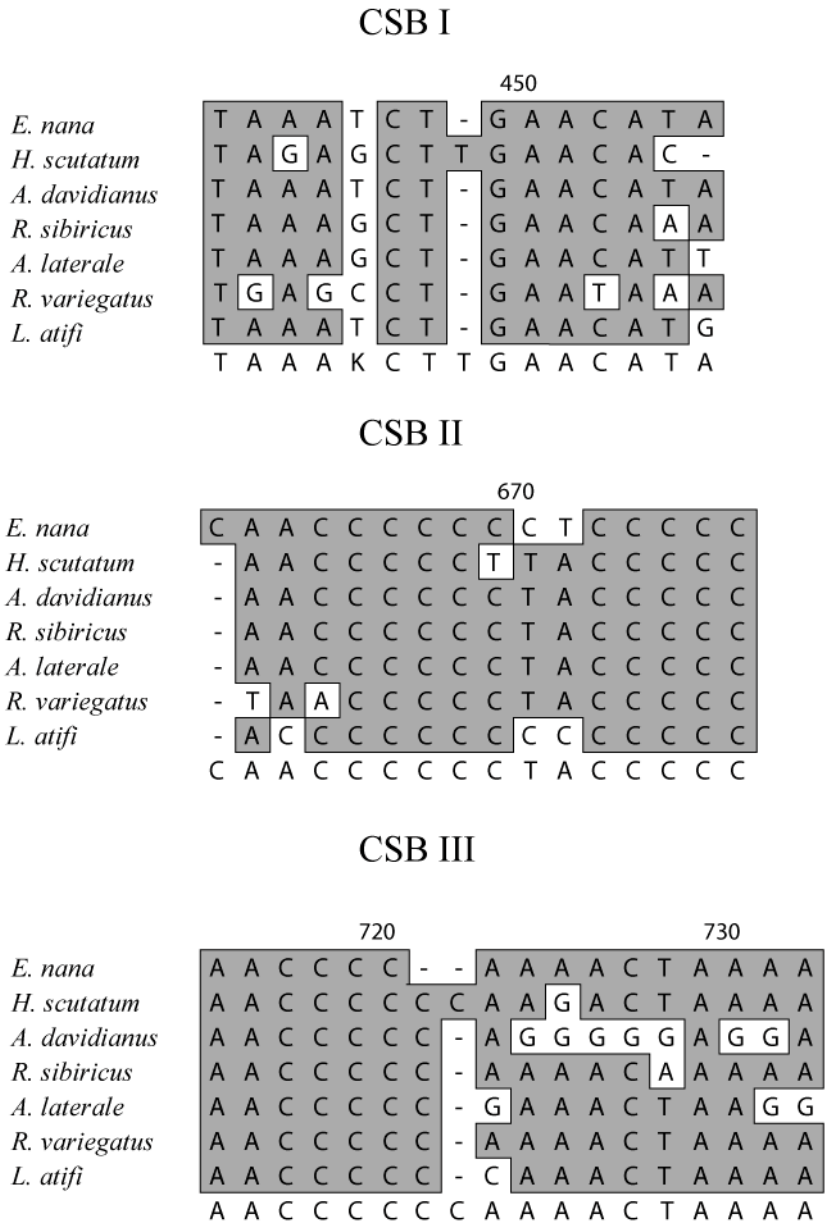

Figure 2.4. Conserved sequence blocks identified in the salamander control region. The identity of CSB I is uncertain, although the consensus motif ACATA is similar to that found in other vertebrate CSB I. Adjacent conserved sites near this motif are shown and may be part of the salamander CSB I.

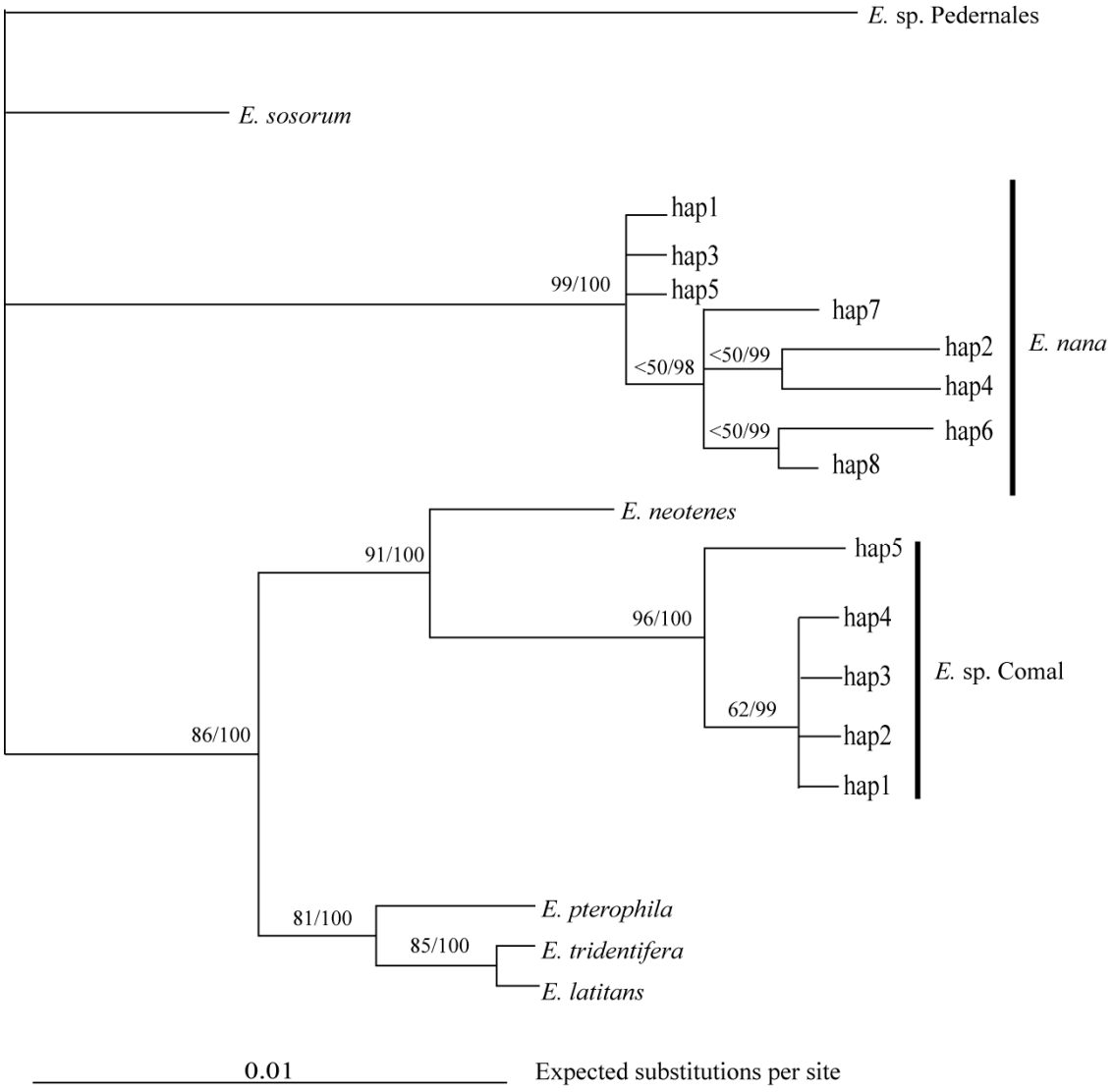

Figure 2.5. Phylogram resulting from Bayesian analysis. The Bayesian analysis was similar in topology to the parsimony analysis, and bootstrap values are mapped to the tree along with posterior probabilities (bootstrap/posterior probability). The commonest haplotypes from *E. sosorum* (Barton Springs), *E. neotenes*, *E. pterophila*, *E. tridentifera*, and *E. latitans* are shown.

CHAPTER 3

SYSTEMATICS, BIOGEOGRAPHY, AND EVOLUTION OF THE SOUTHEASTERN BLEPSIMOLGE

3.1 Introduction

Molecular systematic techniques have been instrumental in the recognition of species diversity within central Texas *Eurycea* and other plethodontids. The identification of five new species and resurrection of two others within the past dozen years has been facilitated by the use of molecular data. Chippindale et al. (1993) described *Eurycea sosorum* from Barton Springs in Austin and Hillis et al. (2001) described *E. waterlooensis* from the same locality. Chippindale et al. (2000) described three new species (*E. tonkawae, E. naufragia,* and *E. chisolmensis*) using cytochrome *b* sequence data and allozyme allele frequencies, in addition to morphology. In the same study, *E. troglodytes, E. pterophila* and *E. latitans* (see Chippindale et al. 2000 for taxonomic history) were resurrected; each had previously been synonymized by Sweet (1978b; 1984) under *E. neotenes* (*E. latitans* was considered a hybrid swarm between *E. neotenes* and *E. tridentifera*).

In addition to describing new species, recent studies using molecular data have also enhanced understanding of the evolutionary and biogeographic history of the central Texas *Eurycea*. Three species (*E. tonkawae, E. naufragia,* and *E. chisolmensis)* form a clade which is sister to the remaining central Texas *Eurycea* (Chippindale et al. 2000).

This deep phylogenetic split is concordant with their geographic distribution north of the Colorado River ("northern group"; Chippindale et al. 2000). Members of the *subgenus Typhlomolge* (*E. rathbuni, E. robusta, and E. waterlooensis*) form a clade sister to all other central Texas *Eurycea* that occur south of the Colorado River (Chippindale et al. 2000; Hillis et al. 2001). In their description of a new blind salamander, *E. waterlooensis*, Hillis et al. (2001) provided further support for the relationships proposed by Chippindale et al. (2000) using cytochrome *b* sequence data. In this paper Hillis et al. (2001) also named the major clades of central Texas *Eurycea* (Figure 3.1). Following are the clade names (in boldface) given by Hillis et al. (2001) and their relationships. **Paedomolge** contains all central Texas *Eurycea*, **Septentriomolge**, the "northern group" of Chippindale et al. (2000), consists of *Eurycea tonkawae*, *E. naufragia, E. chisolmensis*. **Notiomolge**, the sister group to Septentriomolge, contains the following two sister clades: **Typhlomolge**, previously the genus *Typhlomolge* (*E. rathbuni*, *E. robusta*, *E. waterlooensis*) and **Blepsimolge**, the "southeastern," "southwestern," and "San Marcos" groups of Chippindale et al. 2000 excluding Typhlomolge - *E. latitans, E. pterophila, E. nana, E. neotenes, E. tridentifera, E. troglodytes*, and *E. sosorum*.

Blepsimolge can be roughly divided into an eastern group and a western group (Chippindale 2000; Chippindale et al. 2000; Hillis et al. 2001). The western clade contains a large species complex (*E. troglodytes* group) including numerous undescribed species (Chippindale et al. 2000; Hillis personal communication). The eastern Blepsimolge consist of the remaining species named above. The distribution of all central Texas *Eurycea* designated by these groups is shown in Figure 3.2.

3.1.1 Systematics of the Southeastern Blepsimolge

The southeastern Blepsimolge is a problematic group in terms of systematics. Several members of this clade have been the subject of numerous taxonomic revisions, and relationships remain poorly resolved, despite extensive study (e.g. Chippindale et al. 2000; Hillis et al. 2001). This group has undergone a rapid radiation, most likely facilitated by geographic isolation, so even moderately fast-evolving molecular markers have not provided adequate resolution of their phylogeny (e.g., Chippindale et al. 2000 and Hillis et al. 2001). The analysis of Chippindale et al. (2000) which included cytochrome *b* sequences, allozyme frequency data, and morphometrics could not resolve fine-scale relationships among the Blepsimolge. While Hillis et al. (2001) were able to use a much larger sequence of cytochrome *b* (~1100 bp vs. 356 bp in Chippindale et al. 2000), the scope of the study did not include a comprehensive survey of Blepsimolge populations, only representatives of each species. Furthermore, the phylogenetic analysis with cytochrome *b* did not provide uniformly high bootstrap support even among the representative species of southeastern Blepsimolge. This could be a reflection of the speed of evolution of the marker used, incomplete taxon sampling, or both. Despite the major advances in our understanding of the evolution of central Texas *Eurycea* provided by the work of Chippindale et al. (2000), they specifically cited the need for additional research on two species complexes (*E. latitans* and *E. troglodytes* groups) and two potentially undescribed species (*E.* sp. Pedernales and *E.* sp. Comal Springs).

Further complicating our understanding of evolutionary relationships is the wide range of morphological variation in the Blepsimolge, which probably results from

selection, or relaxed selection on features associated with cave vs. surface dwelling. Habitat-specific morphological change in this group has resulted in morphological convergence or parallelism, whereby closely related organisms inhabiting similar habitats exhibit similar morphologies (Wiens et al. 2003). The correlation between morphology and habitat has been particularly problematic with respect to taxonomic identification as well as identifying species relationships within the central Texas *Eurycea*. A prime example of this is the extensive history of taxonomic revisions for *E. rathbuni*, for which detailed reviews of this literature are provided by Chippindale (1995); Chippindale (2000); Chippindale et al. (2000); Potter and Sweet (1981); and Sweet (1978a). Furthermore, Wiens et al. (2003) found that using morphological data in phylogenic reconstruction resulted in the placement of *E. rathbuni* and *E. tridentifera* as sister taxa, which is strongly at odds with the well-supported results from molecular analysis (*Eurycea tridentifera* was placed in the former genus *Typhlomolge* by Wake [1966] but returned to *Eurycea* by Mitchell and Smith [1972]). This demonstration of how morphological convergence may mislead phylogenetic analyses, specifically in the central Texas *Eurycea*, suggests that morphological data used to classify or describe relationships among these salamanders is problematic in many instances.

Within Blepsimolge, there are several examples of what appear to be convergent evolution. Sweet (1984) considered morphologically variable topotypical populations of *E. latitans* and *E. troglodytes* the result of secondary contact between a surface-dwelling species (*E. neotenes*) and a troglobite (*E. tridentifera*). However, Chippindale et al. (2000) did not find evidence for hybridization within these populations, although their

conclusion was based upon limited allozyme frequency data and mt sequences. That genetically distinct populations exhibit similar morphologies implies that convergent evolution may be occurring within the southeastern Blepsimolge clade. However, this conclusion may be premature given that the evidence for genetic distinctiveness among these populations is not particularly strong, and their relationships are not well resolved. An alternative interpretation is that certain populations or species are phenotypically plastic and express morphologies specific to their environment. There now is fairly strong evidence that phenotypic plasticity may help explain morphological diversity in various hypogean fishes, as opposed to the commonly invoked 'regressive evolution' explanation, used to describe relaxation of selection in hypogean taxa (Romero and Green, 2005). Additionally, environmental factors influencing the life history mode and morphology recently have been demonstrated in other members of the genus *Eurycea* (Bonett and Chippindale 2006).

Recently, additional specimens from populations of troglomorphic salamanders have been sampled which are remarkably similar in appearance to *E. tridentifera*, but occur outside its recognized range and are geographically closer to populations of other southeastern Blepsimolge such as *E. pterophila*, *E. neotenes*, and *E. latitans*. These troglobites could be additional populations of *E. tridentifera*, the result of hybridization between epigean and hypogean species, or troglobitic morphs of other Blepsimolge. Resolving the systematic and taxonomic status of this taxon, in addition to others, will be a major step towards understanding the evolution of troglobites, morphological convergence, and phenotypic plasticity.

There also are conservation-related issues for which resolution of species boundaries, diversity and relationships are crucial. As mentioned above, two potentially new species within this group have been suggested: *E.* sp. Pedernales and *E.* sp. Comal Springs (Chippindale 2000; Chippindale et al. 2000) and previously unstudied populations that recently have been sampled may also represent distinct species. Further study is critical for many of these populations (e.g. *E.* sp. Comal Springs) due to anthropogenic threats to their habitat (Chippindale et al. 2000; Chippindale and Price 2005) because resolving their taxonomic status could aid in securing their federal or state protection if warranted.

Also of interest is the biogeographic history of the southeastern Blepsimolge, and how this relates to geologic and hydrologic features of the Edwards Plateau. How does the phylogenetic pattern correspond to known aquifer boundaries? What does the evolution of certain geologic formations (such as the Upper and Lower Glen Rose formations) reveal about the evolution of the species inhabiting that area?

To address all of the issues I have outlined above, here I present a detailed molecular study of the southeastern Blepsimolge using highly variable control region (CR) and intergenic spacer (IGS), cytochrome *b* (Cyt*b*), partial NADH dehydrogenase subunit-2 (ND2), and four tRNA genes of the mt genome. As previously demonstrated in Chapter One, the CR and IGS can be quite variable within and among populations, which is especially useful for resolving fine-scale relationships.

3.2 Materials and Methods

3.2.1 Specimen Collection and Sampling

Salamanders were collected from spring and cave sites within the Edwards Plateau region between 1988 and 2005. Many samples used by Chippindale et al. (2000) are included within this study, in addition to specimens from numerous recently discovered populations. Specimens collected during the course of this study were euthanized with MS-222, and tissues taken according to approved IACUC protocols. The species included in this study, following the revisions given by Chippindale et al. (2000) are: *E. latitans* complex, *E. pterophila, E. nana, E. neotenes, E. tridentifera, E. troglodytes* complex, *E. sosorum, E.* sp. Pedernales, *E.* sp. Comal Springs and *E. rathbuni* (outgroup). Figure 3.3 displays the sampling localities for all focal taxa (southeastern Blepsimolge). Appendix A lists collection localities and museum numbers, if available, of all specimens included in this study. All specimens collected prior to 2003 are deposited in the Texas Natural History Collection (Texas Memorial Museum, University of Texas at Austin). All specimens collected from 2003 to 2006 are deposited in the Amphibian and Reptile Diversity Research Center at the University of Texas at Arlington.

3.2.2 Laboratory Methods

DNA was extracted from muscle or liver tissue using several methods. For all of the tissue samples obtained between 2003 and 2006, DNA was extracted using the DNeasy kit from Qiagen. DNA from specimens collected prior to this primarily was extracted using the STE method described by Hillis et al. (1996) and a modification of

the Chelex extraction method (Walsh et al. 1991), as described in Chippindale et al. (2000).

PCR products were amplified primarily using a generic *Taq* polymerase (New England Biolabs) or Hot Start *ExTaq* (Takara) on MJ Research PTC 200 gradient and PTC 100 thermal cyclers. Amplification for PCR and sequencing was performed using the primers listed in Table 3.1. PCR conditions which yielded the most consistent results were as follows: Reactions consisted of 1-2 µl of dilute DNA (typically 10-50ng of DNA, but sometimes as high as 300 ng), 0.5-1.0 µM of each primer, 0.75 mM dNTPs mix, polymerase buffer (1.5 mM $MgCL_2$), and 1-2U *Taq* (or *ExTaq*) polymerase in a total volume of 20µl. Occasionally, 0.5µl of 100% DMSO was used in PCR reactions if there was difficulty in obtaining amplification. Thermal cycling conditions varied greatly depending on the template of interest and difficulty of amplification. Typical conditions are as follows: Step 1: 96° 3 min; Step 2: Annealing temp. 50° 30 s.; Step 3: 72° 1 min/kb; Step 4: 96° 20 s; Step 5: repeat steps 2-4 (x 30); Step 6: 72° 10min; Step 7: 4° hold. Variations of this profile include a step-up annealing temperature, whereby the first 2 or 3 replications include a 3-5° lower annealing temperature, and then step up to the standard annealing temperature to complete the remaining replications. This was used frequently for IGS/CR amplifications. Touchdown protocols also were used for the IGS/CR amplifications if the step up protocol did not work. This consisted of starting at a higher annealing temperature (typically 55 to60°) and dropping the annealing temperature incrementally until a suitable temperature (5 to 10° lower) was reached to complete the remaining cycles.

PCR products were purified using Qiagen gel extraction or PCR purification kits following the manufacturer's protocol, or if nonspecific products were absent, a combination of exonuclease I and shrimp alkaline phosphatase enzymes (USB) was used to remove the unused single stranded DNA and dNTPs, preventing loss of product that occurs with column-based methods. Primers used for sequencing are reported in Table 3.1. Both strands of each marker were sequenced for moderate (complete overlap was not achieved for heteroplasmic repeats; see Chapter One) to complete overlap for most templates using ABI Big Dye v3.1. Unincorporated products were removed with an ethanol precipitation using 2µl 0.75M sodium acetate and 125mM EDTA. Applied Biosystems 377 and 3130xl automated sequencers were used to obtain the sequence data.

3.2.3 Data Analysis

Raw sequence chromatograms were edited with Sequencher v4.2, v4.3 and v4.5 (Gene Codes Corp.). The ClustalW (Thompson et al. 1994) algorithm implemented in MacVector 7.2.3 (Accelrys) was used to perform multiple alignments of the nucleotide sequences, and alignment gaps within the CR and IGS were adjusted manually. Gaps in the IGS and CR were coded using the simple gap coding method (Simmons and Ochoterena 2000) as implemented by GapCoder (Young and Healy 2003). No gaps were present in the cytochrome *b* or ND2 sequences. Two repeat regions within the IGS were removed from the alignment because this region exhibits substantial intrapopulation variation and heteroplasmy in number of repeats (Chapter One). Sequencing in this region is often very difficult and homology of repeats is impossible to establish (Chapter One).

Separate analyses using several methods were performed for the following alignments: (1) partial ND2 plus adjacent $tRNA^{TRP}$ and partial $tRNA^{ALA}$ (this alignment referred to as ND2); (2) cytochrome *b* (Cyt*b*); (3) $tRNA^{THR}$, IGS, $tRNA^{PRO}$, and CR (IGS/CR); (4) Same as 3, except with coded gaps (IGS/CR+gaps); (5) combined alignment containing alignments 1,2 and 3 (combined1); (6) combined alignment with data sets 1, 2, and 4 (combined2). *Eurycea rathbuni* was chosen as the outgroup for all analyses because it (plus *E. waterlooensis*) is well supported as sister to Blepsimolge in previous molecular studies (Chippindale et al. 2000; Hillis et al. 2001; Wiens et al. 2003).

Paup 4.0b10 (Swofford 2000) using PaupUp graphical interface (Calendini and Martin 2005) was used to calculate parsimony steps and pairwise distances. Maximum parsimony analyses were conducted using TNT (Goloboff et al. 2003b) and/or PAUP. Analyses were conducted with a "traditional" search, TBR branch swapping and 10 random-taxon-addition sequence replicates. Support for nodes was evaluated by non-parametric bootstrapping (Felsenstein 1985) with 1000 pseudoreplicates and 10 random-taxon-addition sequence replicates per replicate in TNT. The effects of invariant characters were evaluated using a Poisson bootstrap on the combined dataset (Harshman 1994; Farris et al. 1999 in Horovitz 1999), and these were determined to have no substantial effect on the results.

Modeltest v3.7 (Posada and Crandall 1998) was used to select the appropriate models of evolution under the Akaike Information Criterion for implementation in Bayesian phylogenetic analyses (Posada and Buckley 2004), with the exception of the coded gaps from the IGS/CR. MrBayes v3.1 (Huelsenbeck and Ronquist 2001; Ronquist

and Huelsenbeck 2003) was used to perform the Bayesian analyses on individual as well as the combined data sets. MrBayes allows for partitioning of data to assign different evolutionary model parameters. Partitioning by codon improved likelihood scores over partitioning by the entire gene, so I employed this scheme for both protein coding regions. The gap-coded regions were treated under the binary evolutionary model implemented in MrBayes. Gap characters were coded as zeros or ones and treated as equivalent to restriction site data.

All Bayesian analyses were run twice (simultaneously as implemented in MrBayes v3.1), with four Markov chains (one cold, three heated) and trees were sampled every 100 generations. Number of generations varied depending upon the data set (Table 3.2). Burn-in was determined after verifying posterior probability stationarity using the *cumulative* command of AWTY (Wilgenbusch et al. 2004). The post-burn-in trees from both runs were combined calculate a majority-rule consensus with a cutoff of 50%.

Additionally, to examine retention of ancestral polymorphism and non-bifurcating relationships, a haplotype network was constructed using statistical parsimony (Templeton et al. 1992) as implemented in TCS (Clement et al. 2000). The combined1 data set was used to construct the haplotype network. Gaps were treated as missing data, and a 95% connection limit was used.

3.3 Results

3.3.1 Analyses

A total of 68 individuals are included in this study (61 were sequenced for all genes, and an additional seven were sequenced for just cytochrome *b*), collected from 43

caves and springs throughout the eastern Edwards Plateau. The total lengths of all alignments are as follows: ND2, 619bp; Cyt*b*, 1,110bp; IGS/CR, 1426bp; IGS/CR+gaps, 1523bp; combined1, 3155bp; combined2, 3252bp (Table 3.2). Uncorrected "p" distances for the combined alignment among all taxa ranged from 0-10% (Table 3.3). Among southeastern Blepsimolge uncorrected "p" distances ranged from 0-3% (Table 3.3). Results from parsimony analyses are summarized in table 3.4.

Model parameters input to MrBayes are reported in Table 3.2 along with all data partitions used, and the corresponding model selected by Modeltest. The Bayesian topologies were mostly congruent with parsimony trees in the ND2 and Cyt*b* data sets. Parsimony and Bayesian analyses differed with the IGS/CR and combined data sets on the placement of several problematic taxa, although these results were not strongly supported in either analysis. For simplicity, results for the individual data partitions (data sets 1-4; Table 3.2) are displayed as Bayesian topologies and branch lengths with posterior probabilities and parsimony bootstrap values at each node (Figures 3.4, 3.5, 3.6, and 3.7). The following abbreviations in bold are used to refer to clades in figures and in text: **N**, *E. neotenes* and closely related populations; **W**, population at White Spring; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **SE**, clade of *E. pterophila* and closely related populations with a southeastern distribution (relative to all *E. pterophila*); **NW**, clade of *E. pterophila* and closely related populations with a northwestern distribution; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E.*

pterophila and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P.

Parsimony and Bayesian analysis of the ND2 data set resulted in the least resolved tree compared to the other analyses (Figure 3.4). Cyt*b* gave marginally better support at some of the deeper nodes in the trees than IGS/CR partitions (including and excluding gap data), and better resolution overall than ND2 (Figure 3.5). Both IGS/CR trees were more resolved than those based on Cyt*b* (Figures 3.6 and 3.7). Including gap data did not improve overall resolution of the tree. The Bayesian analysis differed in its placement of clade SE (Figure 3.8a and 3.8b). Including gaps, clade SE forms a polytomy with clades P, LT, and FT2. Excluding gaps it falls within clade P. Excluding gap data in the parsimony analysis helps resolve a ten clade polytomy (Figure 3.9a and 3.9b). The inclusion of gap data results in more higher-level (deep) polytomies (grey squares in Figure 3.8a and 3.9a) than analyses excluding gap data. This is due to greater homoplasy among indel characters compared to nucleotide characters, particularly among higher level relationships. Intraspecific variation was observed at several indel sites (Chapter One) suggesting that several indel positions evolve too rapidly to be suitable for phylogenetic analysis. Furthermore, no model of evolution exists to my knowledge that corrects for "multiple hits" of gap characters in phylogenetic analyses.

Several polytomies remain unresolved even when excluding gap data (Figures 3.8b and 3.9b). Sometimes spurious relationships can be resolved if a problematic taxon is removed, and the analysis repeated. White Spring was a problematic taxon to place, partly due to long branch lengths (Figure 3.10). However, removal of this taxon from the

combined parsimony analysis did not increase node support and did not alter the relationships among other taxa. Removing Cibolo Creek Spring from the analysis decreased node support, and altered the position of White Spring (Figure 3.11).

In the combined analysis (excluding gaps), three equally parsimonious trees were found. The Bayesian analysis recovered one clade which parsimony did not (clade P; Figure 3.12), and differed in its placement of clades FT1 and W. However, there were no strongly supported incongruencies between the Bayesian and parsimony analyses.

The combined analysis, excluding gaps (Figures 3.12 and 3.13) is the preferred phylogenetic hypothesis because of the (presumed) increased homoplasy caused by gap characters, and will be used in all subsequent references to phylogenetic relationships. Support values for particular nodes are referenced in parentheses; MP is the maximum parsimony bootstrap value and PP is the posterior probability.

The haplotype network did not reveal any deeply nested ancestral polymorphisms (Figure 3.14), indicating that lineage sorting among the major clades probably is complete. Several multifurcating relationships occur, and these generally correspond to polytomies within the parsimony and Bayesian analyses. Homoplasy also is evident as indicated by the presence of loops (because mtDNA does not recombine; loops may indicate recombination with a nuclear marker). Other groups are recovered that are consistent with those of the parsimony and Bayesian analyses. Notably, clade P is recovered in the haplotype network which is consistent with the Bayesian analysis, but this clade was not recovered by parsimony.

3.3.2 Systematics

The phylogenetic pattern from the combined analysis does not correspond completely with currently recognized species boundaries: populations of *E. latitans* do not form a monophyletic group and all *E. tridentifera* appear to be conspecific with or at least nested within topotypical (and other) *E. latitans* (Figures 3.12 and 3.13); *E. pterophila* form a poorly supported monophyletic group in the Bayesian analysis (Figure 3.12) and is not recovered as a monophyletic group under parsimony (Figure 3.13), but is recovered by the haplotype network.

Also apparent from the phylogenetic analysis is the presence of what appears to be evidence of historical gene flow among some geographically distant populations. Two Stealth Cave specimens group with the geographically proximate Sharron Spring within the *E. neotenes* clade, while the other Stealth Cave specimen groups with the LT clade (Figures 3.12, 3.13, 3.14). Furthermore, the two from Jacob's Well cluster with different clades within the *E. pterophila* group (Figures 3.12, 3.13, 3.14). One *E. sosorum* haplotype is sister to *E. nana* (Figures 3.12 and 3.13), which is remarkable considering the geographic distance between Barton Springs and San Marcos Springs (Figure 3.3) and the morphological and allozymic distinctiveness of these taxa (Chippindale 2000; Chippindale et al. 2000).

The combined parsimony and Bayesian trees show that troglomorphic *Eurycea* are paraphyletic (Figure 3.15), and low genetic divergence between troglomorphic and epigean populations (Figure 3.15). One example of this pattern is the phylogenetic position of the Preserve Cave specimen within the *E. pterophila* clade. While certain

members of this clade display varying degrees of troglomorphism (e.g. T Cave; Sweet 1978a), most are epigean and lack the light tan pigmentation, flattened snout and reduced eyes observed in the Preserve Cave population. Preserve Cave is geographically proximate (Figure 16) and very similar in appearance to topotypical *E. tridentifera* (Honey Creek Cave). Classification of taxa novel to this study as "troglomorphic" was done by cursory observation of head shape, eye diameter, pigmentation, and their similarity to other previously designated troglomorphic populations (namely *E. tridentifera*). I therefore provisionally classify animals from Preserve Cave, Camp Bullis Cave #1 and Camp Bullis Cave #3 as "troglomorphic" in the sense that they most resemble nearby populations of *E. tridentifera*. A more detailed assessment of morphological variation of these salamanders is pending.

3.4 Discussion

3.4.1 Systematic Status and Taxonomic Considerations

The paedomorphic salamanders of central Texas have been the subject of a series of taxonomic and systematic revisions beginning after Stejneger (1896) described the first, *Eurycea* (formerly *Typhlomolge*) *rathbuni,* from an artesian well in San Marcos, Hays County. The complete histories of the taxonomic revisions of all members of this group are fairly extensive and would be redundant to repeat here; see Sweet (1978a), Chippindale (1995), Chippindale (2000), and Chippindale et al. (2000) for detailed treatment of the taxonomic history of the central Texas *Eurycea*. Below I address the most recent taxonomic revisions given by Chippindale et al. (2000) relevant to this study, add range extensions where appropriate, and assess the systematic status of each taxon.

3.4.1.1 *Eurycea neotenes*

This species was long considered to be widespread throughout the Edwards Plateau by numerous authors (for reviews, see Sweet 1978a, Chippindale 2000, Chippindale et al. 2000, Chippindale and Price 2005). Based on molecular evidence, Chippindale et al. (2000) dramatically restricted its range to "spring populations from the vicinity of the type locality." My results generally agree with this assessment. Populations sampled in this area (northern Bexar Co. and Medina Co., southern Kendall Co., and eastern Comal Co.) form a well supported monophyletic group and the relationships among these taxa are well resolved. The clade N is sister to the clade consisting of *E. latitans*, *E. tridentifera*, *E. pterophila* and other recently sampled populations (LTP) within the Guadalupe and Blanco River drainages (Figures 3.12, 3.13, 3.14). The epigean salamander at Comal Springs (Comal Co.) which has tentatively been treated as a novel species (Chapter One; Chippindale et al. 2000; Hillis et al. 2001) is sister to that from nearby Hueco Springs, and together these are sister to *E. neotenes*. Given the high support for the phylogenetic position of *E*. sp. Comal Springs and low sequence divergence (0.76%) from topotypical *E. neotenes,* I recommend that this population no longer be considered a novel species but rather a range extension of *E. neotenes.* Furthermore, the distribution of *E. neotenes* should also be extended from Helotes Spring, Bexar Co., Leon Springs, Bexar Co., and Muellller's Spring, Bexar Co. (Chippindale et al. 2000) to include the following populations: Morales Spring, Bexar Co., Lost Dog Spring, Bexar Co., Pecan Springs, Medina Co., Zizelman Spring (a.k.a. Culebra Spring), Bexar Co., Sharron Spring, Bexar Co., and Stealth Cave, Bexar Co. The

latter two populations I assign tentatively given the possibility of introgression with *E. tridentifera* (see discussion below).

3.4.1.2 *Eurycea latitans*

Eurycea latitans was originally described from Cascade Caverns, Kendall Co. (Smith and Potter 1946). Chippindale et al. (2000), who extended the range of *E. latitans* from the type locality (Cascade Caverns) to springs and caves in or near the Cibolo Creek and Guadalupe River drainages, noted that this group was problematic, and labeled it a "catch-all" group (i.e., *E. latitans* complex) for difficult-to-place populations of the southeastern clade. Even the taxonomic status of topotypical *E. latitans* has been disputed. Citing considerable morphological variability within this population, Sweet (1984) regarded it as a hybrid swarm derived from a more widespread surface dweller which he considered *E. neotenes*, and a cave-dweller, *E. tridentifera*. On the basis of allozyme frequency data, Chippindale et al. (2000) concluded that populations of *E. tridentifera* (Honey Creek Cave, Badweather Pit and Ebert Cave, Comal Co.) were exclusive of the population in the Cascade Caverns system and populations that they also assigned to *E. latitans* localities. Contrary to this, my findings show that the Cascade Caverns population (represented here by the subterranean extension, Pfeiffer's Water Cave), other putative *E. latitans*, and *E. tridentifera* (LT) form a well supported monophyletic group (81 MP, >95 PP; Figures 3.12 and 3.13). Additionally, *E. latitans* as recognized by Chippindale et al. (2000) is clearly paraphyletic (Figures 3.12, 3.13 and 3.14). The Rebecca Creek population appears to be conspecific with *E. pterophila*, which is contrary to its placement by Chippindale et al. (2000). Populations from Cibolo

Creek (Kendall Co.), Cherry Creek (Kerr Co.), and Cloud Hollow (Kerr Co.) were previously assigned to *E. latitans* and here do not group with topotypical *E. latitans.* Their relationships to other *E. latitans*, *E. pterophila* and *E. neotenes* populations are not clearly resolved, but they may represent distinct species because they do not group with the other clades within the haplotype network.

3.4.1.3 *Eurycea tridentifera*

Eurycea tridentifera was described by Mitchell and Reddell (1965) and is among the most troglomorphic Texas *Eurycea* outside of the Typhlomolge clade (which consists of *E. rathbuni*, *E. robusta* and *E. waterlooensis*). When examining the taxonomic status of *E. tridentifera*, Chippindale et al. (2000) supported the continued recognition of this taxon citing a chromosomal nondisjunction unique to Badweather Pit and Honey Creek Cave (Bogart 1967), distinctive morphological features (Mitchell and Reddell 1965; Wake 1966; Mitchell and Smith 1972; Sweet 1977, 1978a, and 1984), and similar allozyme allele frequencies. However no unique cytochrome *b* substitutions or fixed unique allozyme alleles were reported and allozyme data supporting this grouping primarily were limited to a single locus for which differences from other populations clearly were not fixed. Despite the evidence presented by the aforementioned authors I do not find any evidence to suggest that *E. tridentifera* is genetically distinct. Mitochondrial sequence divergence (uncorrected "p") based on 3,252 base pairs within the LT group is very low (0-0.2%; Table 3.3) and monophyly of the clade is strongly supported by both parsimony and Bayesian analyses (81 MP, >95 PP; Figures 3.12 and 3.13). The phylogenetic pattern suggests that *E. tridentifera* is likely a troglobitic form of

E. latitans rather than a distinct species. As mentioned above, several morphological variants have been observed or collected from the Cascade Caverns system and Honey Creek Cave (Sweet 1978a, 1984; and Chippindale et al. 2000). The low level of divergence and phylogenetic pattern suggest that there is gene flow among epigean and troglomorphic salamanders within this clade. The haplotypes within this group form several multifurcating relationships, providing further evidence for gene flow among these populations (Figure 3.14).

3.4.1.4 *Eurycea pterophila*

Eurycea pterophila, described from Fern Bank Spring, Hays Co., (Burger et al. 1950) generally occurs in springs and caves within the Blanco River drainage. Sweet (1978a, 1978b) considered this species to be part of what he considered a widespread *E. neotenes* based upon morphological data, and Hamilton (1973) was unable to distinguish this taxon morphologically from other central Texas *Eurycea* populations. Based on molecular evidence and geographic conformity to one river drainage, Chippindale et al. (2000) resurrected the name and extended the range of this species from its type locality to include all populations they studied within the Blanco River drainage: Fern Bank Spring, (Hays Co.), Zercher Spring (Blanco Co.), Boardhouse Springs (Blanco Co.), T Cave, (Blanco Co.), Peavey's Springs (Kendall Co.), and Grapevine Cave (Hays Co.). These populations are included in my study in addition to the following populations also within the Blanco River drainage: White Spring (Blanco Co.), Jacob's Well (Hays Co.), CWAN (Cave Without A Name, Kendall Co.), and Otte's Spring (Hays Co.). All Blanco River drainage populations sampled plus the two Guadalupe River basin populations

form a monophyletic group (with the exception of White Spring) in the Bayesian analysis (66 PP; clade P, Figure 3.12) and in the haplotype network (Figure 3.14), but form a polytomy in the parsimony analysis (Figure 3.13). The phylogenetic position of White Spring is questionable, and it is fairly distinct from other salamanders that I examined, with about 1-3% sequence divergence from other southeastern Blepsimolge. The remaining Blanco River drainage populations form a polytomy (Figures 3.12 and 3.13), but each clade within the polytomy has high posterior probability and bootstrap support. The two largest clades in this region can be described as a northwest (NW) group (Peavey's Spring, T Cave, Zercher Spring, and Boardhouse Springs) and a southeast (SE) group (Grapevine Cave, Rebecca Creek, Preserve Cave, and Jacob's Well). Preserve Cave, which is on the south side of the Guadalupe River and close in proximity to Honey Creek Cave, groups with the SE clade. This is unusual not only because of the location of Preserve Cave (but see *Phylogeographic Patterns* section below) but also because of the resemblance the salamanders have to the Honey Creek Cave animals and members of other cave populations that resemble and are conspecific with *E. tridentifera* (Figure 3.17). Rebecca Creek Spring and Sattler's Deep Pit, also within the Guadalupe River basin, are highly supported as part of the SE *E. pterophila* clade (75 MP, >95 PP) and therefore *E. pterophila* should not be restricted to just the Blanco River drainage.

3.4.1.5 *Eurycea sosorum*

Although it inhabits perhaps the busiest city park and swimming hole in Austin, Texas (and maybe in all of Texas), *E. sosorum* was not formally described until relatively recently (Chippindale et al. 1993). It is now listed as Federally Endangered, and is

managed by the City of Austin in cooperation with the U.S. Fish and Wildlife Service. The range of this epigean species is restricted to four spring outlets at Barton Springs, in Zilker Park, Travis County. The phylogenetic position of this taxon as sister to *E. neotenes, E. latitans, E. pterophila* and *E. tridentifera* and its genetic distinctiveness is well supported by the molecular data (however, see discussion below). Research currently is underway by Bendik and Chippindale to assess the genetic diversity within *E. sosorum* using mt IGS/CR sequence and microsatellites. Of over 50 mt IGS/CR sequences for *E. sosorum* that I have collected so far, a minority of them (fewer than five) group with *E. nana* and Taylor Springs, that latter located midway between Austin and San Marcos. San Marcos Springs is the type locality for *E. nana*. Only one of these unusual haplotypes was included in my analyses to illustrate this recent discovery.

3.4.1.6 *Eurycea nana*

This species was previously known only from San Marcos Springs, Hays County. There had been previous confusion in the literature regarding the presence of *E. nana* at Comal Springs, although this clearly was based on compounded historical errors (Chippindale et al. 1998). Chapter One addresses this subject in part, and it is clear that these two populations are genetically distinct, and are not even sister taxa. The phylogenetic position of *E. nana* as sister to all other southeaster Blepsimolge with the exception of *E.* sp. Pedernales is well supported (Figures 3.12 and 3.13), and it is extremely distinct allozymically (Chippindale 1995; Chippindale et al. 2000). Taylor Springs is a recently discovered population of which little is known. It occurs roughly equidistant between Austin and San Marcos. Although salamanders from this site are

genetically similar to *E. nana*, their coloration and body form are very different (personal observation; Hillis, personal communication). Further study and survey of this population definitely is needed to determine whether gene flow exists between *E. nana* and Taylor Springs.

3.4.1.7 *Eurycea* sp. Pedernales

The existence of this salamander was first recognized by Chippindale et al. (2000) and it is only known from two small springs at Hammett's Crossing along the Pedernales River in Travis Co. Chippindale et al. (2000) suggested this salamander almost certainly represents a distinct species based upon unique allozyme alleles, mitochondrial haplotype, and morphology, and a species description is in preparation. This species is sister to all other southeastern Blepsimolge and has many unique sequence character states. The analyses that I present here are in agreement with the assessment of Chippindale et al. (2000) that this salamander is indeed distinct and should be described as a new species. Unfortunately, three separate attempts by me to collect additional specimens of this salamander at the (previously) only known localities were unsuccessful. Recently another population in the Pedernales River drainage has been found (Andrew Price, personal communication). However, no molecular analysis has been conducted yet.

3.4.2 Introgression and Hybridization

Sweet (1984) suggested that the morphological spectrum he observed in several populations of *Eurycea* was due to hybridization between a widespread epigean form (that he considered *E. neotenes*) and a cave-dwelling species (*E. tridentifera*). Given the

evidence at the time, his conclusions were plausible, but molecular analysis of these putatively hybrid populations failed to support Sweet's hypothesis (Chippindale et al. 2000). *Eurycea latitans* from the type locality (Cascade Caverns, Kendall Co.), which Sweet (1984) considered to be of hybrid origin, was resurrected and distinguished from *E. tridentifera* (Chippindale et al. 2000). The molecular analysis presented here clearly does not support this view; *E. latitans* and *E. tridentifera* are most likely conspecific. Sweet (1984) was probably correct in concluding that topotypical *E. latitans* contained genes shared with *E. tridentifera* (although not because of hybridization), and Chippindale et al. (2000) appear to have been correct in concluding that *E. latitans* is not a hybrid form (although not because it is genetically distinguishable from *E. tridentifera*). However, there appear to be several other instances of hybridization. In this section I give an account of each case and discuss possible mechanisms by which they could occur. First it is necessary to discuss the suitability of mt DNA markers for fine scale phylogenetic studies.

There are several caveats in use of mt DNA as a phylogenetic marker. First, the mode of inheritance typically is maternal; therefore phylogenies will only track the maternal lineage. This can cause a problem in the event of hybridization among putatively distinct groups if gene flow is sex-biased, sample size is small (larger sample sizes give a better chance of detecting both mt haplotypes among hybrids while heterozygotes may be detected with nuclear genes), or an introgressed mitochondrial haplotype takes over the ancestral haplotype (via a selective advantage or drift enhanced by population bottlenecking). However, there is no evidence suggesting sex-biased gene

flow within *Eurycea* to date. Furthermore, two samples or more from each population were sequenced if available, but for several populations only one specimen was available for sequencing. Cases where an introgressed mt haplotype has replaced the original haplotype have been documented by conflicting phylogenies of mtDNA and nuclear DNA sequence data (e.g., in crickets: Shaw 2002; and fishes, e.g., Strecker et al. 2003). A well resolved nuclear phylogeny is not available for the southeastern Blepsimolge, so this comparison cannot be made. Given the extremely low mt divergences among several clades (e.g. P and LT; Figure 3.9), very rapidly evolving molecular markers (e.g. microsatellites; work in progress by Bendik and Chippindale) will be required to test for congruence between nuclear and mt-based relationships. The use of mtDNA in assessing hybridization has some distinct advantages over other markers. The inheritance mode of mtDNA provides a means for identifying the maternal form and evaluating the directionality of introgression (Dowling et al. 1996). The major advantage of using mt markers is that they evolve very rapidly relative to nuclear DNA so that unique haplotypes arise frequently and allow for fine-scale resolution of relationships. Mitochondrial DNA typically does not recombine and has a lower effective population size than nuclear DNA, so that ancestral polymorphisms will not persist as long as those in nuclear genes, and therefore lineage sorting occurs more rapidly (Moore 1995). In recently diverged species, incomplete lineage sorting and hybridization can give the same phylogenetic signal (Avise and Ball 1990). However, the haplotype network suggests that lineage sorting is relatively complete among the major clades (Figure 3.14), with the exception of several ancestral haplotypes within the LT and P clades.

3.4.2.1 Barton Springs

In an ongoing survey of mt CR and IGS variation in *E. sosorum*, several divergent haploypes have been found. Of over 50 mt sequences that have been obtained thus far (unpublished data), several are recovered in phylogenetic analyses grouping with the Taylor Spring population and *E. nana*. This divergent haplotype is sister to the Taylor Springs population, both of which are sister to *E. nana* (Figure 3.12 and 3.13). I have only included one such haplotype in this study to document this phenomenon, and therefore must caution that in no way does this represent haplotype frequencies of the two divergent *E. sosorum* haplotypes.

Given the low frequency of this haplotype (less than 10%), as well as the overall evidence for the distinctiveness of *E. sosorum* (morphological, allozyme allele frequency, mt DNA divergence of the common haplotype; Sweet 1978a; Chippindale et al. 1993; Chippindale et al. 2000; Chapter One), it is likely that this represents introgression of genes from another distinct population due to secondary contact. The closest known population related to the "divergent" haplotype lies roughly between Austin and San Marcos at Taylor Springs. The presence of this divergent haplotype within *E. sosorum* raises several interesting questions. What is the source of this introgression? How long ago did this introgression occur? Is the range of *E. sosorum* much greater than previously thought, such that there is current gene or intermittent flow with a more distant population, such as Taylor Springs or other undiscovered populations? Do these introgressed mitochondria represent an instance (or multiple instances) of past hybridization events? This likely is not a case of ancestral haplotype retention, otherwise

known as incomplete lineage sorting. The mt control region evolves very rapidly and divergences among these distinct haplotypes are high. It is unlikely that a haplotype from an ancestral population could have persisted without change in the population while other haplotypes continued to accumulate mutations (barring strong selection on particular haplotypes).

3.4.2.2 Jacob's Well

Only two specimens from this site (a submerged cave in Hays Co., where collection is extremely difficult) were obtained for this study. One groups with the SE clade and the other with the type locality, Fern Bank Spring, and both clades are well supported (75 MP, >95 PP; 74 MP, >95 PP, respectively; Figures 3.12 and 3.13). This is consistent with the geographic location of Jacob's Well, which lies between Fern Bank Spring (12.5 km) and Grapevine Cave (8 km). This may represent ongoing introgression or incomplete lineage sorting within the *E. pterophila* clade.

3.4.2.3 Stealth Cave

Of the three specimens from Stealth Cave sequenced, two cluster with Sharron Spring and the other specimen groups with LT. These sites are about 600m apart on the Camp Bullis Training Site, north of San Antonio in Bexar Co. Both Sharron and Stealth animals possess a reticulated color pattern typical of *E. neotenes*, although they have much larger bodies and display varying degrees of troglomorphism: The putative hybrid, Stealth Cave 3, appears to have a wider and flatter snout than individuals from Sharron Spring (Figure 3.18). This probably represents introgression of *E. tridentifera* or *E. latitans* (LT) genes into the *E. neotenes* gene pool. Stealth Cave is in a hydrologically

distinct unit from that of Sharron Spring; Sharron Spring is perched above the hydrologic unit within which Stealth Cave is located, and may have limited hydrologic connection to it (George Veni, personal communication). One possibility is that gene flow is unidirectional from Sharron Spring to Stealth Cave and that the Stealth Cave population hybridizes with an undiscovered population of LT (because Stealth Cave specimen 3 does not have an identical haplotype to any sampled localities). However, additional sampling is required to test whether LT haplotypes are present within the Sharron Spring gene pool. The nearest known population of LT is Camp Bullis Cave 3, approximately 9 km north of Stealth Cave (Figure 3.16).

3.4.2.4 Preserve Cave

Salamanders from Preserve Cave are in an interesting geographic and phylogenetic position. Their congeners are in the SE *E. pterophila* clade and are geographically proximate. Superficially, they bear a strong resemblance to *E. tridentifera* from Honey Creek Cave (and other recognized localities for *E. tridentifera*; Figure 3.17), less than 3 km away (Figure 3.16). One possible explanation is that this population has some introgression of SE *E. pterophila* genes but is actually more closely affiliated to the LT clade given its proximity and morphological resemblance to *E. tridentifera*. While this is merely speculation, it is plausible given the evidence for introgression among LT and N (however see discussion below). Unfortunately, I was only able to obtain one specimen from Preserve Cave, although several more specimens have recently been collected.

3.4.3 Phylogeographic Patterns

Linking evolutionary history with geologic and other historical events can be a difficult if not impossible task. The study of biogeography often requires evidence from a variety of disciplines, such as geology, paleontology, geography, ecology, and even climatology (Lomolino et al. 2006). For example, timing of geologic events such as plate tectonics, glaciation, volcanic activity, and fossilization can aid in explaining and timing evolutionary events when coupled with a phylogeny (Patterson 1999). However, even with a multidisciplinary approach, sometimes the precise cause or timing of an evolutionary event can only be roughly estimated. Unfortunately no closely related fossil Texas *Eurycea* have been found and radioisotope dates for aquifer development and cave inundation are sparse. Although one can view the fragmented springs or caves that *Eurycea* inhabit as islands, the complex (and changing) nature of the permeable strata they live in, or on, make elucidating patterns of past and present dispersal difficult. Despite this, below I present a working hypothesis for the biogeographic history of central Texas *Eurycea*, with an emphasis on the southeastern Blepsimolge clade.

Few clear-cut phylogeographic trends exist within Blepsimolge, although two major patterns are apparent. First, the phylogenetic split between the southwestern Blepsimolge (*E. troglodytes* complex sensu Chippindale et al. 2000) and the southeastern Blepsimolge corresponds to the division between the Edwards-Trinity and Trinity Aquifer boundaries (Figure 3.19). This split also roughly corresponds to the division between the exposed Edwards and Glen Rose limestone formations. Although sampling for the southwestern *E. troglodyes* complex in this study is minimal, they are highly

supported as sister to the southeastern Blepsimolge in other more comprehensive studies (Chippindale et al. 2000; Hillis personal communication; and unpublished nuclear RAG-1 sequence data). Second, the most divergent Blepsimolge appear to be distributed on the periphery of the Edwards Plateau. *Eurycea* sp. Pedernales, sister to all southeastern Blepsimolge, occurs at the northern edge of their distribution along the Pedernales River in an isolated patch of Cow Creek Limestone. *Eurycea nana* and *E. sosorum* are found along the far eastern edge of the range of central Texas *Eurycea,* in an area of the Edwards Limestone formation known as the Balcones Escarpment. *Eurycea neotenes* (here, including *E.* sp. Comal Springs and Hueco Springs), is sister to the remaining taxa and inhabits springs of the Balcones Escarpment in Bexar and Comal Counties, occupying the southeasternmost distribution of central Texas *Eurycea*.

The deepest phylogenetic splits within the southeastern Blepsimolge, and other central Texas *Eurycea*, correspond to vicariant events that seem to have taken place along the periphery of their current distribution. The oldest split among central Texas *Eurycea* corresponds to the division of the northern (Septentriomolge) and southern (Notiomolge) clades by the Colorado River, a major geographic barrier and one of the oldest features of the Edwards Plateau (Chippindale 1995 and references therein; Chippindale et al. 2000). The distribution of Septentriomolge is restricted to the northern segment of the Edwards fault zone aquifer (as distinguished from the Edwards-Trinity Aquifer) springs and caves (Chippindale et al. 2000).

The next major split occurred between the Typhlomolge and the Blepsimolge clades. Typhlomolge and Blepsimolge probably diverged due to a shift in habitat; the

former occupying the cave or underground niche and the latter occupying the surface spring niche. Although no empirical evidence exists to support that hypothesis, Typhlomolge exhibit extreme cave-associated morphologies and, with the exception of *E. robusta* (of which little is known; Potter and Sweet 1981), all species are sympatric or parapatric with a surface-dwelling counterpart. Their distribution is also along the Balcones Escarpment and at major discharge points of the Edwards Aquifer. The relationships of each species pair are correlated (Figure 3.20), and the phylogenetic splits among the representative southeastern Blepsimolge occur relatively deep within the tree (Figures 3.12 and 3.13). Near-simultaneous speciation of Typhlomolge and Blepsimolge may have occurred in these areas of the Edwards Aquifer (Hillis et al. 2001).

Several major vicariant events that established distinct lineages of the southeastern Blepsimolge probably occurred within the Edwards fault zone springs and caves. These correlations between deep phylogenetic splits and current distribution are suggestive of the importance and influence that Edwards Aquifer development had on the evolution of central Texas *Eurycea*. Faulting during the late Early Miocene (Young 1972) had a major impact on the formation of the Edwards Aquifer, controlling cavern development (Woodruff and Abbott 1979), and producing major discharge points at San Marcos, Comal and Barton Springs. Figure 3.21 depicts the relationships among the southeastern Blepsimolge and their distribution with respect to the Edwards and Trinity Aquifers. The large springs in the Edwards Aquifer, and the aquifer itself, may have provided a persistently stable habitat throughout the history of this group. If the most recent common ancestor of *Eurycea* inhabiting the Edwards Aquifer was once

widespread throughout it, faulting or other disruptions cutting off connections within the aquifer, and consequently limiting or ceasing gene flow among *Eurycea*, may have been the cause of cladogenesis and speciation in the early history of the southeastern Blepsimolge.

Several other biogeographic patterns within the southeastern Blepsimolge are apparent. Species and clade boundaries are not demarcated by river basins; they are more closely correlated with geologic features. *Eurycea latitans* and *E. tridentifera* are distributed throughout the Cibolo Creek basin and to the south side of the Guadalupe River. The range of *E. pterophila*, restricted to the Blanco River drainage by Chippindale et al. (2000), actually extends into the Guadalupe basin as well. The area inhabited by the southeastern Blepsimolge can be characterized by three major geologic formations: the Edwards group and the Upper and Lower Glen Rose formations. The distribution of *E. pterophila*, *E. latitans*, and *E. tridentifera* occurs primarily in the karstified Lower Glen Rose (Figure 3.22), and *E. neotenes* inhabits the periphery of this area to the south, mostly within the Edwards Limestone region.

The most recent common ancestor of N and LTP (Figures 3.12 and 3.13) may have been distributed throughout the now exposed Glen Rose formations prior to cladogenesis. Following events of erosion, possibly cutting off hydrologic connections among populations, divergence among clades ensued. The vicariant event separating N from LTP was probably the exposure of the Lower Glen Rose and subsequent aquifer and cavern development within this region. This is consistent with their current distribution; *E. neotenes* are mostly restricted to Edwards Limestone along the Balcones escarpment.

The least divergent but most widely distributed group, the LTP clade, inhabits the Blanco, Guadalupe, and Cibolo drainages. Relationships between the P and LT clade are unresolved and divergence between these groups is low. This is a reflection of a very rapid radiation within this group. Diversification of this group probably occurred during karstification of the Lower Glen Rose formation in the Guadalupe and Cibolo watersheds (1.3ma to 990 ka B.P.; Veni 1994) which would have allowed for dispersal and vicariance through newly formed caves and springs in this region.

The LT clade has a seemingly disjunct distribution, primarily occurring in caves and springs of the Cibolo Sinkhole Plain but extending northward towards the Guadalupe River into Honey Creek Cave and Knee Deep Cave. This distribution may be explained by a water pirating route from Cibolo Creek to the Guadalupe River through Honey Creek Cave which was established in the early stages of the evolution of the Honey Creek Cave system, approximately 1.2 ma B.P. (Veni 1994). Several populations belonging to this clade do not occur within the Lower Glen Rose karst, but in an isolated patch of the Fort Terrett formation (part of the Edwards group). Less Ranch Spring is adjacent to the boundary of the Fort Terrett and Upper Glen Rose formations and Bear Creek spring occurs in the Fort Terrett formation and in a drainage basin opposite other *E. latitans*, although extremely close to the Cibolo Creek Spring locality. Because locality data were estimated from 7.5 minute quadrats as opposed to modern GPS technology for these populations, it is possible that the locations are slightly inaccurate. However, two other somewhat anomalous groups occur in this region as well. The position of the Cibolo Creek Spring clade (FT2) is poorly resolved in the phylogenetic

tree, forming a polytomy with White Spring (W), LTP and FT2 in the Bayesian analysis, and sister to N and LTP in the parsimony analysis. Cloud Hollow and Cherry Creek (FT1) are monophyletic and part of a polytomy with P and LT. It seems that several cladogenic events are correlated with the erosion and possible separation of hydrologic units within this Fort Terrett "island." Further resolution of the phylogeny would likely benefit from the addition of *Eurycea* from localities in this area.

The SE clade is distributed throughout patches of the Lower Glen Rose karst along the Blanco and Guadalupe River basins (Figure 3.22). Members of the NW clade inhabit springs and caves near the Blanco River, and occur within two minor aquifers associated with the Llano uplift. Their distribution along the Blanco River is correlated with a historical dispersal pattern from west to east. Peavey's Spring, the most divergent population is also the westernmost, followed by Zercher Spring, etc.

Preserve Cave is very close in proximity to Knee Deep Cave and Honey Creek Cave and animals from this population phenotypically resemble Honey Creek Cave *E. tridentifera* (and other *E. tridentifera*). Preserve Cave and Knee Deep Cave are both in the Cow Creek Limestone and drain into the south side of the Guadalupe River, but the Preserve Cave population is well supported as within the SE *E. pterophila* clade. While this may represent an introgressed *E. pterophila* haplotype in an *E. tridentifera* population (see *Introgression* section), there are several reasons why the Preserve Cave population may be genetically isolated from the other nearby Guadalupe River basin caves. First, Preserve Cave occurs in a different formation than Honey Creek Cave and there are no known hydrological connections between the two. Second, a study done on

genetic relationships of isopods found a similar pattern: Preserve Cave populations of *Lirceolus* did not group with the nearby Knee Deep Cave population (Krejca 2005). Instead, Preserve Cave *Lirceolus* was sister to western populations of isopods (Krejca 2005). Although this relationship is not congruent with *Eurycea* phylogeography (as Preserve Cave *Eurycea* do not group with the western clade [*E. troglodytes* complex]), this does emphasize the unusual distribution of diverse organisms from Preserve Cave.

White Spring is another anomalous population. It occurs in the middle of the distribution of *E. pterophila* (clade P), is highly divergent, and its phylogenetic position is somewhat uncertain given its conflicting placement by both parsimony and Bayesian analyses. Given its location in the Lower Glen Rose karst, near the division between Lower and Upper Glen Rose formations, it would be expected to fall within clade P. It is difficult to surmise how White Spring became isolated from its neighbors without further knowledge of current and past hydrology in this region. This population may represent a distinct species and further investigation is required.

3.4.4 Evolution of Cave-Associated Features

Cave-dwelling in animals has been of fundamental interest to biologists for numerous reasons (Culver 1982; Wilkens et al. 2000). Animals in total darkness must cope with a variety of challenges which shape aspects of their behavior, ecology and evolution (Langecker 2000). One particularly prevalent topic regarding the evolution of hypogean fauna has been the processes which lead to the reduction or loss of phenotypic characters. The term “regressive evolution” has been used to describe the loss or reduction of traits that are biologically useless in an environment absent of light, such as

loss of pigmentation or eye function (Romero and Green 2005). These traits in particular are highly variable within various species and populations of the central Texas *Eurycea* (e.g. Sweet 1984). Numerous hypogean populations demonstrate no apparent loss of pigmentation or eye size compared to their surface counterpart while others exhibit what could be considered extreme troglomorphism. Conversely, several predominantly surface populations share traits commonly observed in subterranean species (e.g. *E. sosorum*, *E. chisolmensis*).

Troglomorphism within the central Texas *Eurycea* appears to have evolved independently many times. Typhlomolge (*E. rathbuni*, *E. robusta* and *E. waterlooensis*) all display what appears to be an "advanced" troglomorphic morphotype. For example, these include pale or silvery pigmentation, extremely reduced eyes, elongated limbs, reduced trunk size but larger bodies. Initially placed in the genus *Typhlomolge* because of their unique morphology, they were subsequently synonymized under *Eurycea* based upon molecular evidence (Chippindale et al. 2000). *Eurycea tridentifera* had also been assigned to the genus *Typhlomolge* based on morphological characters (Wake 1966) but later was transferred back to *Eurycea* (Mitchell and Smith 1972), a move supported by molecular data (Chippindale et al. 2000). Wiens et al. (2003) demonstrated that morphologies of *E. rathbuni* (Typhlomolge) and *E. tridentifera* (Blepsimolge) are convergent and this can mislead a phylogenetic analysis based solely upon morphology. Blepsimolge and Septentriomolge exhibit a wide spectrum of morphological variation among and within species. For example, populations of *E. tonkawae* (Septentriomolge) in the Buttercup Creek Cave system include individuals with various degrees of

depigmentation, eye reduction, and troglomorphic head morphology (personal observation). Several groups of the Blepsimolge are extremely variable in morphology; e.g. Cascade Caverns, Honey Creek Cave, and Valdina Farms Sinkhole (Sweet 1984).

Several questions emerge regarding the evolution of troglomorphism in the southeastern Blepsimolge. Is the evolution of "advanced" troglomorphism time-dependent, whereby "older" hypogean populations display more exaggerated cave-associated features than those that invaded the cave habitat more recently? Did troglomorphism evolve independently (and, therefore, convergently) within the southeastern Blepsimolge and, if so, how many times? Are some species and populations more phenotypically plastic than others with respect to troglomorphic traits?

Sweet (1984) suggested that the caves within the Cibolo Sinkhole Plain and northern Bexar Co. (which encompass the distributions of *E. tridentifera* and many populations of *E. latitans*) are among the oldest in the plateau region, which would have allowed a long time for the evolution of cave-associated features. The low divergences among troglomorphic and epigean southeastern Blepsimolge indicate that evolution of cave-associated features can occur relatively rapidly. Caves of the Cibolo basin are probably much younger than those of the Guadalupe basin (Veni 1994), so the first troglobites probably inhabited the caves of the Honey Creek group. This puts an upper bound for the evolution of troglomorphism no later than 1.2ma B.P., which is the earliest estimated date for the development of the Honey Creek Cave system (Veni 1994). Rapid evolution of troglomorphism, specifically eye reduction and depigmentation, has also

been documented in the Mexican cave tetra, *Astyanax mexicanus*. The radiation of this group is thought to be of Pleistocene age (Mitchell et al. 1977).

The phylogenetic positions of troglomorphic salamanders within the southeastern Blepsimolge are consistent with a pattern of morphological convergence or parallelism, whereby species in similar habitats (or exposed to the same selective pressures) independently evolve similar dimensions of traits (Wiens et al. 2003). This pattern can be observed by comparing the phylogenetic position of Preserve Cave (clade SE) and *E. tridentifera* (clade LT; Figure 3.15). Less clear is the origin of troglomorphism within the LT clade (Figure 3.15). According to the haplotype analysis (Figure 3.14), Badweather Pit 2 (*E. tridentifera*) haplotype is ancestral to haplotypes present within other troglomorphic populations and epigean populations. One possible interpretation of this pattern is that cave-associated characters are evolving faster than lineage sorting. If these cave-associated traits can evolve at such a rapid pace, there is no reason why troglomorphism could not have arisen independently several times within this clade. However, given the presence of numerous cave populations that do not appear to exhibit extreme cave-associated morphologies (e.g. Knee Deep Cave, CWAN, Jacob's Well, T Cave, Pfeiffer's Water Cave) this does not seem like a plausible explanation unless these caves are very young or salamanders invaded them recently. Alternatively, balancing selection may be acting within LT, preserving both epigean and troglobitic morphologies within the population. Finally, the evolution of cave-associated features may have arisen independently within LT and later undergone introgressive hybridization with conspecific surface dwelling populations. This would similar to the scenario proposed by Sweet

(1984) for Cascade Caverns (which exhibited a spectrum of morphologies), although he considered the surface and cave forms to be distinct species.

That cave-associated features seem to have evolved independently multiple times among Typhlomolge and Blepsimolge (Wiens et al. 2003) and among Preserve Cave and clade LT within the central Texas *Eurycea* is not surprising. It is relatively common among cave fauna to evolve similar morphologies given the selective environment (or relaxed selection) in a cave habitat. The most obvious of these traits would be reduced eyes and loss of dark body pigments which is seen in almost all orders of vertebrates with troglobitic representatives (Weber 2000). The heads of numerous species of troglobitic fishes are often comparatively large with a depressed shovel-like snout (Weber 2000) which is not unlike those of troglobitic salamanders such as *E. tridentifera*, members of the subgenus Typhlomolge, the cave-dwelling plethodontid *Haideotriton wallacei* (now widely considered a member of *Eurycea*), and the proteid *Proteus anguinus*. Convergent evolution has also been documented in a recent radiation of Mexican cavefish, the *Astyanax mexicanus* group (Wilkens and Strecker 2003; Strecker et. al. 2004), whereby populations of blind and depigmented fish do not group to the exclusion of surface eyed and pigmented populations.

Convergence is not the only plausible explanation for the phylogenetic pattern of troglomorphic *Eurycea*. Alternatively, these species may exhibit phenotypic plasticity of troglomorphic traits. Evidence from studies of the Mexican cavefish *Astyanax* suggests that some aspects of eye development are phenotypically plastic. When blind and depigmented larvae were reared under light conditions they had more advanced optic

structures than those reared in darkness (Romero et al. 2002). There is no evidence to suggest that the Typhlomolge are phenotypically plastic for their troglomorphic state; there are no known epigean species or populations within this clade, and the captive individuals that have been raised in daylight do not exhibit evidence of reverting to a surface-morph (Chippindale and Price 2005). However, the picture is less clear for southeastern Blepsimolge. Several populations exhibit a range of morphotypes (e.g. Honey Creek Cave system and Cascade Caverns Cave system; Sweet 1984) which appear to be conspecific. Divergence is very low among all populations of the LT clade. Overall pairwise distances (IGS/CR) are lower for LT (n=15; 0-0.2%) than for *E. nana* (n=24; 0-0.7%) and Comal Springs (n=9; 0-0.3%) populations. The implication is that either troglomorphic traits such as eye degeneration, depigmentation, and various characters relating to head shape can respond to selection extremely rapidly or that these traits (or developmental processes which control expression of these traits) arise in response to environmental cues. Borowsky and Wilkens (2002) have demonstrated through QTL mapping that troglomorphic evolution in *Astyanax mexicanus* might be facilitated by pleiotropy or genetic hitchhiking. Pleiotropy could potentially provide a means for regulation of multiple troglomorphic traits at once. Hitchhiking would allow for selection of constructive traits and "regressive" ones simultaneously (if they are in proximity on the chromosome).

Rapid selection for specific traits, balancing selection, phenotypic plasticity, or some combination of these factors may explain the phylogenetic pattern of troglomorphic *Eurycea* presented here. The field for addressing these questions and others regarding the

evolution of troglomorphism in the central Texas *Eurycea* is wide open. Unfortunately little is known about the breeding habits of most central Texas *Eurycea* (but see Roberts et al. 1995; Fries 2002), so experiments such as a common garden or others that require a captive colony potentially could be difficult. Despite this challenge, there is much to be gained in terms of our understanding of natural selection and development of vertebrates by examining the evolution of troglomorphism in central Texas *Eurycea*. Research aimed at examining the impact of phenotypic plasticity, evidence supporting or rebuking the notion of 'regressive evolution,' and selection for troglomorphic traits can be informed by the model system of the cave tetra *Astyanax mexicanus*. However, because the central Texas *Eurycea* exhibit troglomorphism at several phylogenetic levels, each with varying degrees, they provide a more intriguing and more powerful system for answering fundamental questions about the evolution of cavernicolous organisms and the processes of natural selection.

3.5 Conclusions

The results of this study should enhance the ability of researchers to address other questions about the evolution of central Texas *Eurycea*. The molecular markers presented here will undoubtedly be useful for resolving a detailed phylogeny of the *E. troglodytes* complex and of the Septentriomolge. The phylogenetic results provide a framework within which to test hypotheses regarding the evolution and development of cave-associated features in troglobitic organisms.

There remain many unresolved questions regarding the evolution of the central Texas *Eurycea* in general, and the southeastern Blepsimolge in particular. Development

of microsatellite markers for *E. sosorum* currently is underway and likely will be useful for further analysis of the genetic structure of the southeastern Blepsimolge. This will also enable the reconstruction of a nuclear phylogeny with which to test the hypotheses presented here. Furthermore, resolution of the relationships among southeastern Blepsimolge will be enhanced by including additional localities. There are numerous populations documented by Sweet (1978a) for which genetic material is not available (Figure 3.23), and future studies of this group should focus on gaining access to these localities, documenting the status of those populations, and collecting additional specimens. The continuation of research on this group of salamanders is necessary not only for their potential as a system for addressing broad evolutionary questions, but also to gain better understanding and greater protection and conservation of the aquifer waters they inhabit. The spring and cave salamanders of central Texas occupy a fragile and unique habitat shared by numerous other endemic species and they represent a fascinating natural experiment in progress.

3.6 Tables

Table 3.1. List of primers used during this study; their nucleotide sequence, position, and direction (forward=heavy strand, reverse=light strand). *indicates primer was only used for sequencing, and not PCR.

Primer Name	Sequence	Position	Direction	Target
Blep12SDL1r	5'-GTGGCTGGCACGAGATTTAC-3'	12SrRNA	reverse	IGS/CR
Blep12SDL2r	5'-GGTGGCTGGCACGAGATT-3'	12SrRNA	reverse	IGS/CR
BlepCBDL1f	5'-CATYGCCGACACACTAGYACTTAC-3'	Cyt*b*	forward	IGS/CR
EurCB5	5'-CCMTTYATTGAAATTGGACAAG-3'	Cyt*b*	forward	IGS/CR
EurDL11*	5'-CTCGCTTGAAAAACGTGTTGTGC-3'	D-loop	reverse	IGS/CR
EurDL2r/c*	5'-ATGCATATGTTAKTTCCAGGG-3'	D-loop	forward	IGS/CR
EurDL3*	5'-GTTAGTCCATAGATTCAAACCAG-3'	D-loop	reverse	IGS/CR
EurDL5r/c*	5'-CACTACTGACACCCAAAGC-3'	D-loop	forward	IGS/CR
PGLU	5’-GAARAAYCANTRTTGTATTCAAC-3’	GLU	forward	Cyt*b*
HEM CB1-5’	5’-CCATCCAACATCTCAGCATGATGAAA-3’	Cyt*b*	forward	Cyt*b*
EuTXThrR	5’-GYCAATGTTTTTCTAAACTACAACAGCATC-3’	THR	reverse	Cyt*b*
METf L4437	5'-AAGCTTTTGGGCCCATACC-3'	MET	forward	ND2
COIr H5934	5'-TGCCAATATCTTTGTGATTTGTT-3'	COI	reverse	ND2
ND2f L5002*	5'-AATCAACCACAAATCCGAAAAAT-3'	ND2	forward	ND2
ASNr H5692*	5'-TTAGGTATTTAGCTGTTAA-3'	ASN	reverse	ND2

Table 3.2. Data alignments and partitions used in Bayesian and parsimony analyses. Grey blocks represent alignment and indented blocks are partitions of each alignment.

Alignment/partition	Length (bp)	Mean base frequencies: A, C, G, T				AIC selected model	MCMC generations	Burn-in (mcmc generations)	Sample Freq	Nst	Rates
Cyt*b*	1110	0.30	0.25	0.13	0.32		1 x 10^6	3 x 10^5	100	n/a	n/a
codon pos1	370	0.30	0.22	0.22	0.27	K80+I				2	equal
codon pos2	370	0.20	0.26	0.14	0.40	HKY				2	equal
codon pos3	370	0.39	0.27	0.05	0.29	GTR				6	gamma
ND2	619	0.37	0.24	0.09	0.30		5 x 10^6	1 x 10^6	100	n/a	n/a
codon pos1	207	0.39	0.22	0.15	0.24	K80				2	equal
codon pos2	206	0.21	0.28	0.08	0.43	HKY				2	equal
codon pos3	206	0.49	0.24	0.05	0.22	HKY				2	equal
Trp and Ala	142	0.36	0.21	0.15	0.28	JC				1	equal
IGS/CR	1426	0.36	0.23	0.15	0.26		5 x 10^6	1.5 x 10^6	100	n/a	n/a
Thr, IGS, Pro	635	0.36	0.24	0.14	0.26	HKY+G				2	gamma
Dloop	791	0.36	0.23	0.16	0.25	HKY+I+G				2	gamma
IGS/CR+gaps	1523	0.36	0.23	0.15	0.26	n/a	5 x 10^6	1.5 x 10^6	100	n/a	n/a
gaps	97	n/a				n/a				1	equal
Combined1	3155	0.34	0.24	0.13	0.29	n/a	5 x 10^6	1.5 x 10^6	100	n/a	n/a
Combined2	3252	0.34	0.24	0.13	0.29	n/a	5 x 10^6	1.5 x 10^6	100	n/a	n/a

Table 3.3. Uncorrected "p" distances for all sequence data combined.

		1	2	3	4	5
Pedernales Spring	1					
Honey Creek Cave 1	2	0.0264				
Rebecca Creek Spring	3	0.0257	0.0046			
Barton Springs 1	4	0.0274	0.0127	0.0124		
Sattler's Deep Pit	5	0.0257	0.0036	0.0019	0.0114	
Preserve Cave	6	0.0262	0.0046	0.0026	0.0128	0.0019
Zizelman Springs 2	7	0.0304	0.0101	0.0108	0.0156	0.0095
Leon Springs	8	0.0284	0.0088	0.0092	0.0143	0.0086
Grapevine Cave	9	0.0254	0.0042	0.0010	0.0121	0.0016
Badweather Pit 1	10	0.0257	0.0013	0.0038	0.0120	0.0035
Jacobs Well 2	11	0.0251	0.0036	0.0013	0.0114	0.0010
Helotes Spring	12	0.0290	0.0088	0.0099	0.0146	0.0086
Sharron Spring	13	0.0294	0.0085	0.0095	0.0149	0.0083
Less Ranch	14	0.0261	0.0020	0.0044	0.0127	0.0041
Knee Deep Cave	15	0.0264	0.0013	0.0047	0.0127	0.0038
Peavey's Spring	16	0.0297	0.0091	0.0089	0.0172	0.0089
Comal Springs	17	0.0294	0.0105	0.0114	0.0162	0.0102
Hueco Springs	18	0.0297	0.0095	0.0098	0.0162	0.0089
White Spring	19	0.0329	0.0117	0.0129	0.0200	0.0122
Cloud Hollow Spring	20	0.0267	0.0049	0.0048	0.0138	0.0048
Honey Creek Cave Spring	21	0.0264	0.0007	0.0038	0.0124	0.0035
Cherry Creek Spring	22	0.0280	0.0062	0.0060	0.0149	0.0060
Mueller's Spring	23	0.0290	0.0098	0.0098	0.0146	0.0092
Cibolo Creek Spring 1	24	0.0267	0.0065	0.0063	0.0136	0.0063
Camp Bullis Cave#3 1	25	0.0261	0.0020	0.0044	0.0130	0.0035
Stealth Cave 3	26	0.0254	0.0016	0.0035	0.0117	0.0032
T Cave 1	27	0.0294	0.0078	0.0073	0.0168	0.0070
Boardhouse Springs 2	28	0.0297	0.0085	0.0082	0.0178	0.0076
Fern Bank Springs	29	0.0254	0.0042	0.0029	0.0133	0.0035
Pfeiffer's Water Cave 2	30	0.0261	0.0016	0.0041	0.0124	0.0038
San Marcos Springs	31	0.0306	0.0199	0.0181	0.0204	0.0181
Ebert Cave 1	32	0.0257	0.0013	0.0039	0.0123	0.0036
Taylor Springs	33	0.0290	0.0186	0.0168	0.0191	0.0168
Bear Creek Spring	34	0.0260	0.0016	0.0041	0.0117	0.0038
Zercher Spring	35	0.0287	0.0068	0.0067	0.0162	0.0060
Lost Dog Spring	36	0.0287	0.0091	0.0092	0.0140	0.0086
Otte's Spring 1	37	0.0254	0.0036	0.0029	0.0127	0.0029
Honey Creek Cave 2	38	0.0258	0.0007	0.0038	0.0118	0.0029
Zizelman Spring 1	39	0.0308	0.0098	0.0105	0.0153	0.0093
CWAN 2	40	0.0260	0.0042	0.0035	0.0133	0.0035
Boardhouse Springs 1	41	0.0287	0.0075	0.0073	0.0168	0.0067
Badweather Pit 2	42	0.0254	0.0010	0.0035	0.0117	0.0032
Cibolo Creek Spring 2	43	0.0254	0.0052	0.0051	0.0124	0.0051
Cibolo Creek Spring 3	44	0.0254	0.0046	0.0051	0.0121	0.0045
T Cave 2	45	0.0291	0.0075	0.0073	0.0169	0.0070
Ebert Cave 2	46	0.0254	0.0016	0.0041	0.0117	0.0038

Table 3.3 - *Continued.*

		1	2	3	4	5
Pfeiffer's Water Cave 1	47	0.0260	0.0010	0.0041	0.0120	0.0032
Pecan Springs	48	0.0297	0.0108	0.0102	0.0160	0.0096
Less Ranch Spring 1	49	0.0260	0.0016	0.0041	0.0125	0.0038
Camp Bullis Cave#3 2	50	0.0258	0.0016	0.0041	0.0127	0.0032
Stealth Cave 1	51	0.0294	0.0085	0.0095	0.0149	0.0083
Stealth Cave 2	52	0.0294	0.0085	0.0095	0.0149	0.0083
Otte's Spring 1	53	0.0264	0.0039	0.0038	0.0133	0.0032
CWAN 1	54	0.0260	0.0042	0.0035	0.0134	0.0035
Camp Bullis Cave#1	55	0.0257	0.0016	0.0041	0.0127	0.0032
Fessenden Springs	56	0.0811	0.0783	0.0790	0.0782	0.0788
Barton Springs 2	57	0.0303	0.0209	0.0194	0.0210	0.0188
Morales Springs	58	0.0303	0.0095	0.0105	0.0149	0.0092
Jacob's Well 1	59	0.0248	0.0046	0.0035	0.0124	0.0032
E. rathbuni	60	0.0935	0.0922	0.0916	0.0913	0.0913
Carson Cave	61	0.0842	0.0815	0.0798	0.0811	0.0788

Table 3.3 - *Continued.*

	6	7	8	9	10	11	12	13
7	0.0102							
8	0.0099	0.0054						
9	0.0022	0.0105	0.0089					
10	0.0045	0.0099	0.0083	0.0035				
11	0.0016	0.0099	0.0086	0.0010	0.0032			
12	0.0099	0.0067	0.0025	0.0095	0.0089	0.0089		
13	0.0096	0.0070	0.0029	0.0092	0.0086	0.0086	0.0016	
14	0.0051	0.0105	0.0089	0.0041	0.0013	0.0038	0.0095	0.0092
15	0.0048	0.0102	0.0092	0.0044	0.0016	0.0038	0.0092	0.0089
16	0.0093	0.0140	0.0134	0.0086	0.0086	0.0083	0.0137	0.0137
17	0.0115	0.0080	0.0070	0.0111	0.0105	0.0105	0.0076	0.0080
18	0.0096	0.0073	0.0070	0.0095	0.0092	0.0092	0.0070	0.0067
19	0.0123	0.0148	0.0155	0.0125	0.0122	0.0119	0.0148	0.0145
20	0.0055	0.0113	0.0094	0.0045	0.0042	0.0042	0.0100	0.0097
21	0.0045	0.0099	0.0089	0.0035	0.0013	0.0035	0.0089	0.0086
22	0.0067	0.0124	0.0108	0.0057	0.0054	0.0054	0.0115	0.0111
23	0.0102	0.0048	0.0045	0.0095	0.0089	0.0092	0.0064	0.0067
24	0.0070	0.0111	0.0095	0.0060	0.0057	0.0057	0.0102	0.0098
25	0.0045	0.0099	0.0089	0.0041	0.0019	0.0035	0.0089	0.0086
26	0.0041	0.0102	0.0086	0.0032	0.0010	0.0029	0.0092	0.0089
27	0.0074	0.0121	0.0115	0.0070	0.0073	0.0064	0.0118	0.0115
28	0.0083	0.0124	0.0121	0.0079	0.0082	0.0073	0.0121	0.0118
29	0.0041	0.0102	0.0086	0.0025	0.0035	0.0029	0.0092	0.0089
30	0.0048	0.0102	0.0086	0.0038	0.0010	0.0035	0.0092	0.0089
31	0.0195	0.0233	0.0216	0.0178	0.0191	0.0181	0.0223	0.0220
32	0.0046	0.0101	0.0081	0.0036	0.0006	0.0032	0.0088	0.0084
33	0.0182	0.0220	0.0204	0.0165	0.0178	0.0168	0.0210	0.0207
34	0.0048	0.0102	0.0086	0.0038	0.0010	0.0035	0.0092	0.0089
35	0.0067	0.0114	0.0111	0.0063	0.0067	0.0057	0.0111	0.0108
36	0.0099	0.0041	0.0038	0.0089	0.0083	0.0086	0.0057	0.0060
37	0.0035	0.0099	0.0083	0.0025	0.0029	0.0022	0.0089	0.0086
38	0.0038	0.0093	0.0083	0.0035	0.0006	0.0029	0.0083	0.0080
39	0.0100	0.0010	0.0058	0.0102	0.0096	0.0096	0.0070	0.0073
40	0.0041	0.0105	0.0089	0.0032	0.0035	0.0029	0.0095	0.0092
41	0.0073	0.0114	0.0111	0.0070	0.0073	0.0064	0.0111	0.0108
42	0.0041	0.0095	0.0079	0.0032	0.0003	0.0029	0.0086	0.0083
43	0.0057	0.0099	0.0083	0.0047	0.0044	0.0044	0.0089	0.0086
44	0.0051	0.0092	0.0083	0.0048	0.0044	0.0041	0.0083	0.0080
45	0.0074	0.0124	0.0118	0.0070	0.0073	0.0064	0.0121	0.0118
46	0.0048	0.0102	0.0086	0.0038	0.0010	0.0035	0.0092	0.0089

Table 3.3 - *Continued.*

	6	7	8	9	10	11	12	13
47	0.0041	0.0095	0.0086	0.0038	0.0010	0.0032	0.0086	0.0083
48	0.0097	0.0038	0.0061	0.0099	0.0099	0.0093	0.0080	0.0083
49	0.0048	0.0102	0.0086	0.0038	0.0010	0.0035	0.0093	0.0089
50	0.0042	0.0095	0.0086	0.0038	0.0016	0.0032	0.0086	0.0083
51	0.0096	0.0070	0.0029	0.0092	0.0086	0.0086	0.0016	0.0000
52	0.0096	0.0070	0.0029	0.0092	0.0086	0.0086	0.0016	0.0000
53	0.0038	0.0102	0.0092	0.0035	0.0038	0.0029	0.0092	0.0089
54	0.0042	0.0105	0.0089	0.0032	0.0035	0.0029	0.0096	0.0093
55	0.0042	0.0096	0.0086	0.0038	0.0016	0.0032	0.0086	0.0083
56	0.0783	0.0790	0.0779	0.0787	0.0787	0.0778	0.0773	0.0777
57	0.0202	0.0240	0.0227	0.0191	0.0207	0.0185	0.0230	0.0233
58	0.0105	0.0064	0.0035	0.0102	0.0095	0.0095	0.0038	0.0041
59	0.0038	0.0099	0.0086	0.0032	0.0041	0.0025	0.0089	0.0092
60	0.0902	0.0923	0.0928	0.0913	0.0919	0.0904	0.0925	0.0934
61	0.0780	0.0817	0.0813	0.0795	0.0811	0.0789	0.0803	0.0803

Table 3.3 - *Continued.*

	14	15	16	17	18	19	20	21
15	0.0016							
16	0.0092	0.0092						
17	0.0111	0.0108	0.0144					
18	0.0099	0.0095	0.0124	0.0076				
19	0.0129	0.0122	0.0152	0.0155	0.0151			
20	0.0048	0.0048	0.0091	0.0120	0.0107	0.0132		
21	0.0019	0.0016	0.0086	0.0105	0.0089	0.0122	0.0048	
22	0.0060	0.0063	0.0102	0.0131	0.0118	0.0145	0.0016	0.0060
23	0.0095	0.0098	0.0140	0.0083	0.0083	0.0161	0.0103	0.0095
24	0.0057	0.0067	0.0111	0.0118	0.0105	0.0135	0.0068	0.0063
25	0.0025	0.0022	0.0089	0.0105	0.0092	0.0122	0.0048	0.0019
26	0.0016	0.0019	0.0089	0.0108	0.0095	0.0125	0.0045	0.0016
27	0.0080	0.0079	0.0064	0.0137	0.0105	0.0135	0.0084	0.0076
28	0.0089	0.0086	0.0077	0.0140	0.0108	0.0145	0.0093	0.0082
29	0.0041	0.0044	0.0083	0.0108	0.0092	0.0122	0.0045	0.0035
30	0.0003	0.0013	0.0089	0.0108	0.0096	0.0126	0.0045	0.0016
31	0.0197	0.0200	0.0239	0.0226	0.0223	0.0261	0.0203	0.0190
32	0.0013	0.0013	0.0088	0.0104	0.0094	0.0123	0.0042	0.0013
33	0.0185	0.0188	0.0227	0.0213	0.0210	0.0248	0.0190	0.0178
34	0.0016	0.0019	0.0083	0.0102	0.0095	0.0119	0.0045	0.0016
35	0.0073	0.0070	0.0061	0.0130	0.0098	0.0129	0.0077	0.0067
36	0.0089	0.0092	0.0134	0.0076	0.0076	0.0155	0.0097	0.0089
37	0.0035	0.0038	0.0083	0.0105	0.0092	0.0119	0.0039	0.0035
38	0.0013	0.0010	0.0083	0.0099	0.0086	0.0116	0.0042	0.0006
39	0.0102	0.0099	0.0135	0.0083	0.0077	0.0146	0.0110	0.0096
40	0.0038	0.0044	0.0089	0.0111	0.0099	0.0125	0.0042	0.0041
41	0.0079	0.0076	0.0067	0.0130	0.0098	0.0135	0.0084	0.0073
42	0.0010	0.0013	0.0083	0.0102	0.0089	0.0119	0.0039	0.0010
43	0.0051	0.0054	0.0099	0.0105	0.0092	0.0122	0.0055	0.0051
44	0.0051	0.0048	0.0096	0.0099	0.0086	0.0116	0.0055	0.0044
45	0.0080	0.0080	0.0064	0.0140	0.0105	0.0139	0.0084	0.0076
46	0.0016	0.0019	0.0083	0.0102	0.0095	0.0119	0.0045	0.0016
47	0.0010	0.0006	0.0086	0.0102	0.0089	0.0119	0.0045	0.0010
48	0.0106	0.0109	0.0145	0.0096	0.0080	0.0165	0.0113	0.0106
49	0.0010	0.0013	0.0090	0.0109	0.0096	0.0125	0.0045	0.0016
50	0.0022	0.0019	0.0086	0.0102	0.0089	0.0119	0.0045	0.0016
51	0.0092	0.0089	0.0137	0.0080	0.0067	0.0145	0.0097	0.0086
52	0.0092	0.0089	0.0137	0.0080	0.0067	0.0145	0.0097	0.0086
53	0.0044	0.0041	0.0089	0.0108	0.0089	0.0122	0.0048	0.0038
54	0.0038	0.0045	0.0090	0.0112	0.0099	0.0125	0.0042	0.0041
55	0.0022	0.0019	0.0086	0.0102	0.0089	0.0119	0.0045	0.0016
56	0.0788	0.0794	0.0809	0.0783	0.0783	0.0851	0.0781	0.0791
57	0.0214	0.0214	0.0246	0.0236	0.0236	0.0271	0.0216	0.0207
58	0.0102	0.0098	0.0137	0.0083	0.0080	0.0155	0.0106	0.0095
59	0.0044	0.0048	0.0086	0.0105	0.0099	0.0126	0.0052	0.0044
60	0.0924	0.0926	0.0944	0.0949	0.0921	0.0944	0.0926	0.0923
61	0.0811	0.0817	0.0839	0.0824	0.0806	0.0859	0.0803	0.0814

Table 3.3 - *Continued.*

	22	23	24	25	26	27	28	29
23	0.0114							
24	0.0079	0.0105						
25	0.0060	0.0095	0.0063					
26	0.0057	0.0092	0.0060	0.0022				
27	0.0095	0.0121	0.0099	0.0076	0.0076			
28	0.0105	0.0127	0.0108	0.0083	0.0086	0.0025		
29	0.0057	0.0092	0.0060	0.0035	0.0038	0.0067	0.0076	
30	0.0057	0.0092	0.0054	0.0022	0.0013	0.0076	0.0086	0.0038
31	0.0216	0.0229	0.0197	0.0197	0.0187	0.0230	0.0239	0.0190
32	0.0055	0.0091	0.0058	0.0019	0.0010	0.0075	0.0084	0.0036
33	0.0204	0.0217	0.0184	0.0185	0.0175	0.0217	0.0226	0.0178
34	0.0057	0.0092	0.0060	0.0022	0.0013	0.0076	0.0086	0.0038
35	0.0089	0.0118	0.0092	0.0067	0.0070	0.0016	0.0022	0.0060
36	0.0108	0.0006	0.0098	0.0089	0.0086	0.0115	0.0121	0.0086
37	0.0051	0.0089	0.0054	0.0035	0.0032	0.0064	0.0073	0.0025
38	0.0054	0.0089	0.0057	0.0013	0.0010	0.0070	0.0076	0.0035
39	0.0121	0.0038	0.0112	0.0096	0.0099	0.0115	0.0118	0.0099
40	0.0054	0.0095	0.0057	0.0041	0.0038	0.0070	0.0079	0.0032
41	0.0095	0.0118	0.0098	0.0073	0.0076	0.0016	0.0010	0.0067
42	0.0051	0.0086	0.0054	0.0016	0.0006	0.0070	0.0079	0.0032
43	0.0067	0.0092	0.0019	0.0051	0.0048	0.0086	0.0095	0.0048
44	0.0067	0.0092	0.0019	0.0045	0.0048	0.0083	0.0089	0.0048
45	0.0096	0.0124	0.0099	0.0077	0.0076	0.0016	0.0026	0.0067
46	0.0057	0.0092	0.0060	0.0022	0.0013	0.0076	0.0086	0.0038
47	0.0057	0.0092	0.0060	0.0016	0.0013	0.0073	0.0079	0.0038
48	0.0125	0.0061	0.0112	0.0106	0.0102	0.0122	0.0128	0.0099
49	0.0057	0.0093	0.0061	0.0022	0.0013	0.0077	0.0086	0.0038
50	0.0057	0.0092	0.0060	0.0003	0.0019	0.0073	0.0079	0.0032
51	0.0111	0.0067	0.0099	0.0086	0.0089	0.0115	0.0118	0.0089
52	0.0111	0.0067	0.0099	0.0086	0.0089	0.0115	0.0118	0.0089
53	0.0060	0.0098	0.0063	0.0038	0.0041	0.0070	0.0076	0.0035
54	0.0054	0.0096	0.0057	0.0041	0.0038	0.0070	0.0080	0.0032
55	0.0057	0.0092	0.0060	0.0003	0.0019	0.0073	0.0079	0.0032
56	0.0798	0.0786	0.0778	0.0785	0.0787	0.0812	0.0811	0.0785
57	0.0230	0.0239	0.0213	0.0207	0.0204	0.0237	0.0246	0.0201
58	0.0121	0.0054	0.0111	0.0095	0.0098	0.0121	0.0124	0.0095
59	0.0064	0.0092	0.0063	0.0038	0.0038	0.0070	0.0079	0.0013
60	0.0936	0.0932	0.0913	0.0920	0.0916	0.0911	0.0917	0.0916
61	0.0818	0.0816	0.0791	0.0805	0.0814	0.0829	0.0828	0.0805

Table 3.3 - *Continued.*

	30	31	32	33	34	35	36	37
31	0.0194							
32	0.0010	0.0191						
33	0.0182	0.0025	0.0178					
34	0.0013	0.0194	0.0010	0.0181				
35	0.0070	0.0223	0.0068	0.0210	0.0070			
36	0.0086	0.0223	0.0084	0.0210	0.0086	0.0111		
37	0.0032	0.0190	0.0029	0.0178	0.0032	0.0057	0.0083	
38	0.0010	0.0191	0.0006	0.0179	0.0010	0.0060	0.0083	0.0029
39	0.0099	0.0237	0.0098	0.0224	0.0099	0.0109	0.0032	0.0096
40	0.0035	0.0197	0.0036	0.0184	0.0038	0.0063	0.0089	0.0025
41	0.0076	0.0229	0.0074	0.0216	0.0076	0.0013	0.0111	0.0063
42	0.0006	0.0187	0.0003	0.0175	0.0006	0.0063	0.0079	0.0025
43	0.0048	0.0184	0.0045	0.0172	0.0048	0.0079	0.0086	0.0041
44	0.0048	0.0185	0.0045	0.0172	0.0048	0.0073	0.0086	0.0041
45	0.0077	0.0230	0.0065	0.0217	0.0076	0.0016	0.0118	0.0064
46	0.0013	0.0187	0.0010	0.0175	0.0006	0.0070	0.0086	0.0032
47	0.0006	0.0194	0.0010	0.0181	0.0013	0.0063	0.0086	0.0032
48	0.0103	0.0234	0.0101	0.0222	0.0102	0.0118	0.0054	0.0093
49	0.0006	0.0195	0.0010	0.0182	0.0013	0.0070	0.0086	0.0032
50	0.0019	0.0194	0.0016	0.0181	0.0019	0.0063	0.0086	0.0032
51	0.0089	0.0220	0.0084	0.0207	0.0089	0.0108	0.0060	0.0086
52	0.0089	0.0220	0.0084	0.0207	0.0089	0.0108	0.0060	0.0086
53	0.0038	0.0200	0.0039	0.0188	0.0041	0.0060	0.0092	0.0010
54	0.0035	0.0198	0.0036	0.0185	0.0038	0.0064	0.0089	0.0025
55	0.0019	0.0194	0.0016	0.0182	0.0019	0.0064	0.0086	0.0032
56	0.0792	0.0829	0.0778	0.0823	0.0790	0.0807	0.0783	0.0778
57	0.0211	0.0067	0.0204	0.0048	0.0210	0.0230	0.0233	0.0201
58	0.0099	0.0236	0.0094	0.0223	0.0098	0.0114	0.0048	0.0095
59	0.0041	0.0197	0.0042	0.0185	0.0044	0.0064	0.0086	0.0032
60	0.0925	0.0952	0.0919	0.0952	0.0916	0.0913	0.0928	0.0913
61	0.0809	0.0866	0.0810	0.0859	0.0811	0.0828	0.0813	0.0801

Table 3.3 - *Continued.*

	38	39	40	41	42	43	44	45
39	0.0090							
40	0.0035	0.0102						
41	0.0067	0.0109	0.0070					
42	0.0003	0.0093	0.0032	0.0070				
43	0.0045	0.0099	0.0048	0.0086	0.0041			
44	0.0038	0.0093	0.0048	0.0080	0.0041	0.0006		
45	0.0070	0.0119	0.0070	0.0016	0.0070	0.0086	0.0080	
46	0.0010	0.0099	0.0038	0.0076	0.0006	0.0048	0.0048	0.0076
47	0.0003	0.0093	0.0038	0.0070	0.0006	0.0048	0.0041	0.0073
48	0.0099	0.0042	0.0099	0.0118	0.0096	0.0099	0.0099	0.0122
49	0.0010	0.0096	0.0038	0.0077	0.0006	0.0048	0.0048	0.0077
50	0.0010	0.0093	0.0038	0.0070	0.0013	0.0048	0.0041	0.0073
51	0.0080	0.0074	0.0092	0.0108	0.0083	0.0086	0.0080	0.0118
52	0.0080	0.0074	0.0092	0.0108	0.0083	0.0086	0.0080	0.0118
53	0.0032	0.0099	0.0035	0.0067	0.0035	0.0051	0.0044	0.0070
54	0.0035	0.0103	0.0000	0.0070	0.0032	0.0048	0.0048	0.0070
55	0.0010	0.0093	0.0038	0.0070	0.0013	0.0048	0.0041	0.0073
56	0.0784	0.0784	0.0791	0.0801	0.0784	0.0774	0.0767	0.0804
57	0.0204	0.0244	0.0207	0.0236	0.0204	0.0201	0.0198	0.0237
58	0.0089	0.0054	0.0102	0.0114	0.0092	0.0098	0.0092	0.0124
59	0.0038	0.0096	0.0035	0.0070	0.0038	0.0054	0.0051	0.0073
60	0.0918	0.0920	0.0913	0.0907	0.0916	0.0913	0.0910	0.0915
61	0.0807	0.0821	0.0805	0.0818	0.0808	0.0788	0.0786	0.0830

Table 3.3 - *Continued.*

	46	47	48	49	50	51	52	53
47	0.0013							
48	0.0102	0.0102						
49	0.0013	0.0006	0.0102					
50	0.0019	0.0013	0.0103	0.0019				
51	0.0089	0.0083	0.0083	0.0089	0.0083			
52	0.0089	0.0083	0.0083	0.0089	0.0083	0.0000		
53	0.0041	0.0035	0.0102	0.0042	0.0035	0.0089	0.0089	
54	0.0038	0.0038	0.0099	0.0038	0.0038	0.0093	0.0093	0.0035
55	0.0019	0.0013	0.0103	0.0019	0.0000	0.0083	0.0083	0.0035
56	0.0790	0.0788	0.0775	0.0792	0.0788	0.0777	0.0777	0.0784
57	0.0204	0.0207	0.0231	0.0208	0.0204	0.0233	0.0233	0.0207
58	0.0098	0.0092	0.0080	0.0099	0.0092	0.0041	0.0041	0.0098
59	0.0044	0.0041	0.0099	0.0045	0.0035	0.0092	0.0092	0.0038
60	0.0922	0.0919	0.0929	0.0926	0.0917	0.0934	0.0934	0.0919
61	0.0814	0.0811	0.0806	0.0812	0.0808	0.0803	0.0803	0.0808

	54	55	56	57	58	59	60	61
55	0.0038							
56	0.0791	0.0789						
57	0.0205	0.0204	0.0821					
58	0.0102	0.0092	0.0780	0.0243				
59	0.0035	0.0035	0.0779	0.0195	0.0092			
60	0.0916	0.0917	0.1033	0.0977	0.0937	0.0914		
61	0.0802	0.0805	0.0698	0.0869	0.0813	0.0805	0.1021	

Table 3.4. Results from parsimony analysis by data set.

Data Set	Parsimony informative sites	Number of trees	Number of steps
(1) ND2	54	1	142
(2) Cyt*b*	104	1	282
(3) IGS/CR	111	12	402
(4) IGS/CR+gaps	148	1	555
(5) combined1	269	6	826
(6) combined2	306	50	982

Table 3.5. Haplotype codes used in the haplotype analysis, Figure 14.

Specimen	Haplotype	Group
Badweather Pit 1	1	LT
Badweather Pit 2	2	LT
Bear Creek	3	LT
Boardhouse Springs 1	4	P
Boardhouse Springs 2	5	P
Camp Bullis Cave #1	6	LT
Camp Bullis Cave #3 1	7	LT
Camp Bullis Cave #3 2	6	LT
Cave Without A Name 1	8	P
Cave Without A Name 2	8	P
Cherry Creek Spring	9	FT2
Cibolo Creek Spring 1	10	FT1
Cibolo Creek Spring 2	11	FT1
Cibolo Creek Spring 3	12	FT1
Cloud Hollow Spring	13	FT2
Comal Springs	14	N
Ebert Cave 1	15	LT
Ebert Cave 2	16	LT
Fern Bank Spring 1	17	P
Grapevine Cave	18	P
Helotes Spring	19	LT
Honey Creek Cave 1	20	LT
Honey Creek Cave 2	21	LT
Honey Creek Cave Spring	22	LT
Hueco Springs	23	N
Jacob's Well 1	24	P
Jacob's Well 2	25	P
Knee Deep Cave	26	LT
Leon Springs	27	N
Less Ranch Spring 1	28	LT
Less Ranch Spring 2	29	LT
Lost Dog Spring	30	N
Morales Springs	31	N
Mueller's Spring	32	N
Otte's Spring 1	33	P
Otte's Spring 2	34	P
Peavey's Springs	35	P
Pecan Springs	36	N
Pfeiffer's Water Cave 1	37	LT
Pfeiffer's Water Cave 2	38	LT
Preserve Cave	39	P
Rebecca Creek Spring	40	P
Sattler's Deep Pit	41	P
Sharron Spring	42	N
Stealth Cave 1	43	N
Stealth Cave 2	43	N
Stealth Cave 3	43	LT

Table 3.5 – *Continued.*

Specimen	Haplotype	Group
T Cave 1	44	P
T Cave 2	45	P
Zercher Spring	46	P
Zizelman Spring 1	47	N
Zizelman Spring 2	48	N

3.7 Figures

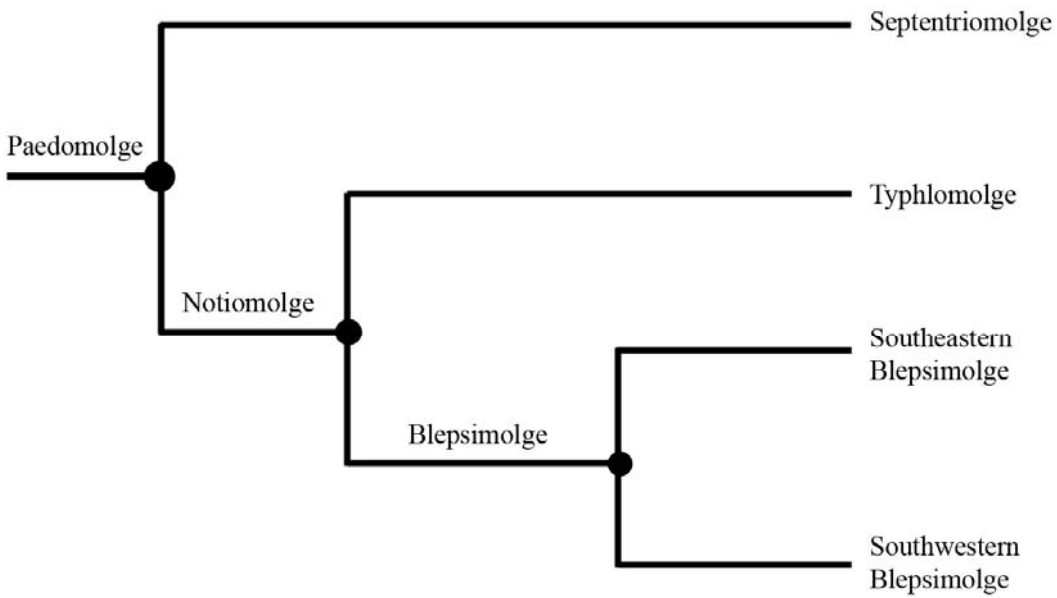

Figure 3.1. Phylocode nomenclature for the central Texas *Eurycea*. Modified from Hillis et al. (2001).

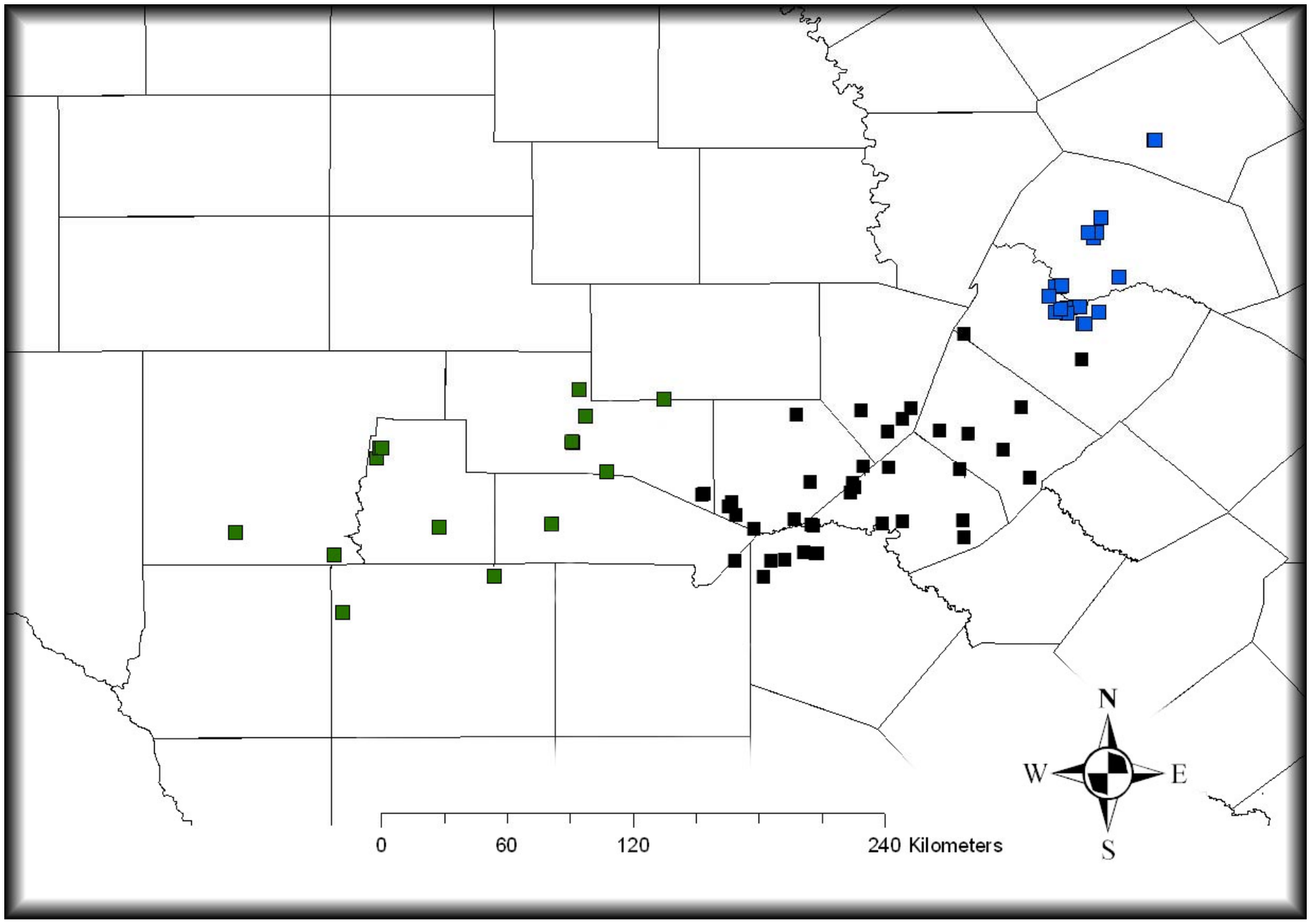

Figure 3.2. Distribution of central Texas *Eurycea* designated by the Phylocode nomenclature. Septentriomolge are indicated by blue squares, the southeastern Blepsimolge are indicated by black squares and occur sympatrically with the Typhlomolge along the eastern border of their distribution. The southwestern Blepsimolge are indicated by green squares.

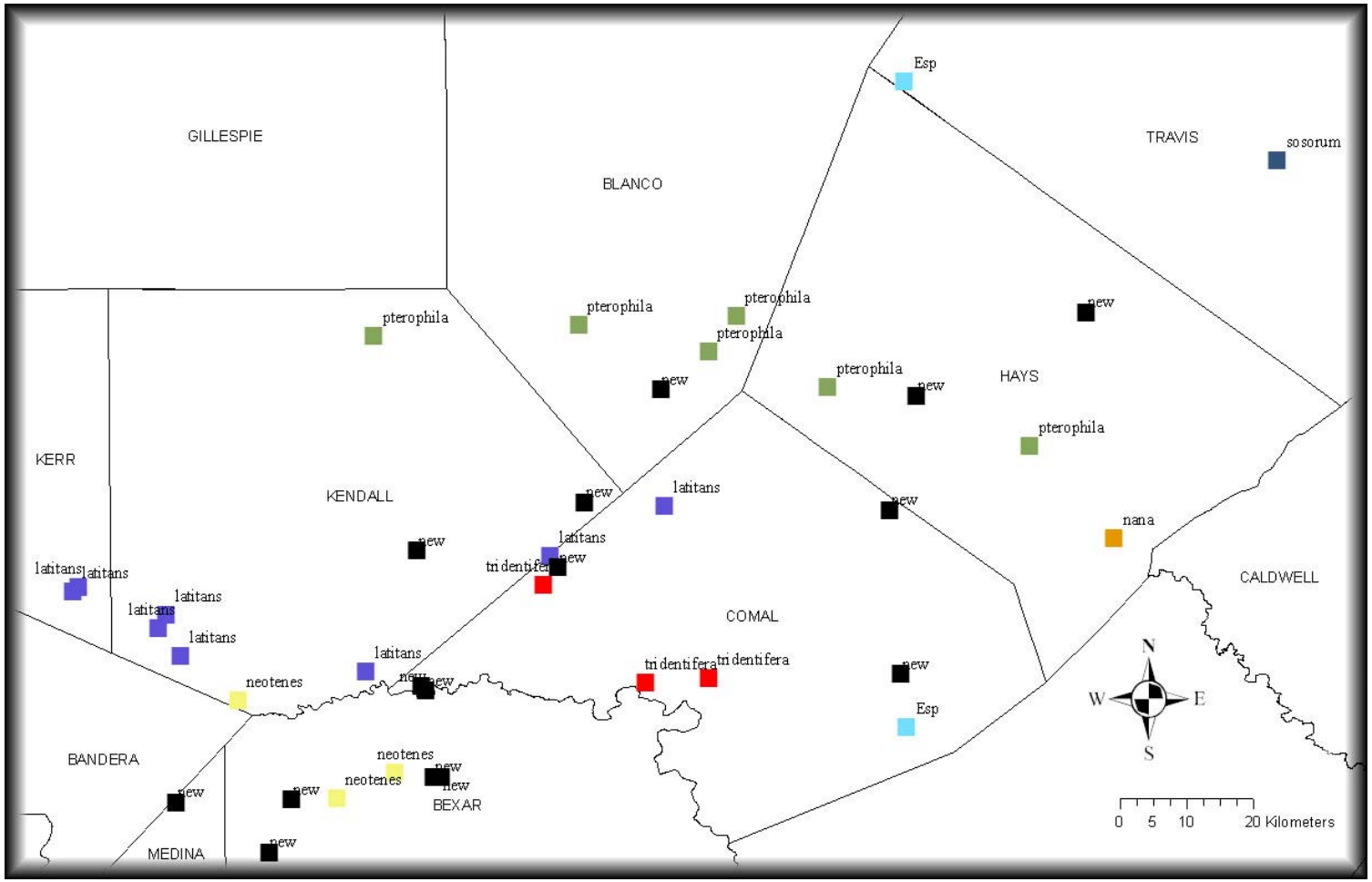

Figure 3.3. Current species designations and localities. Populations assigned to species are indicated with a distinct color. Localities novel to this study are the black squares labeled “new.”

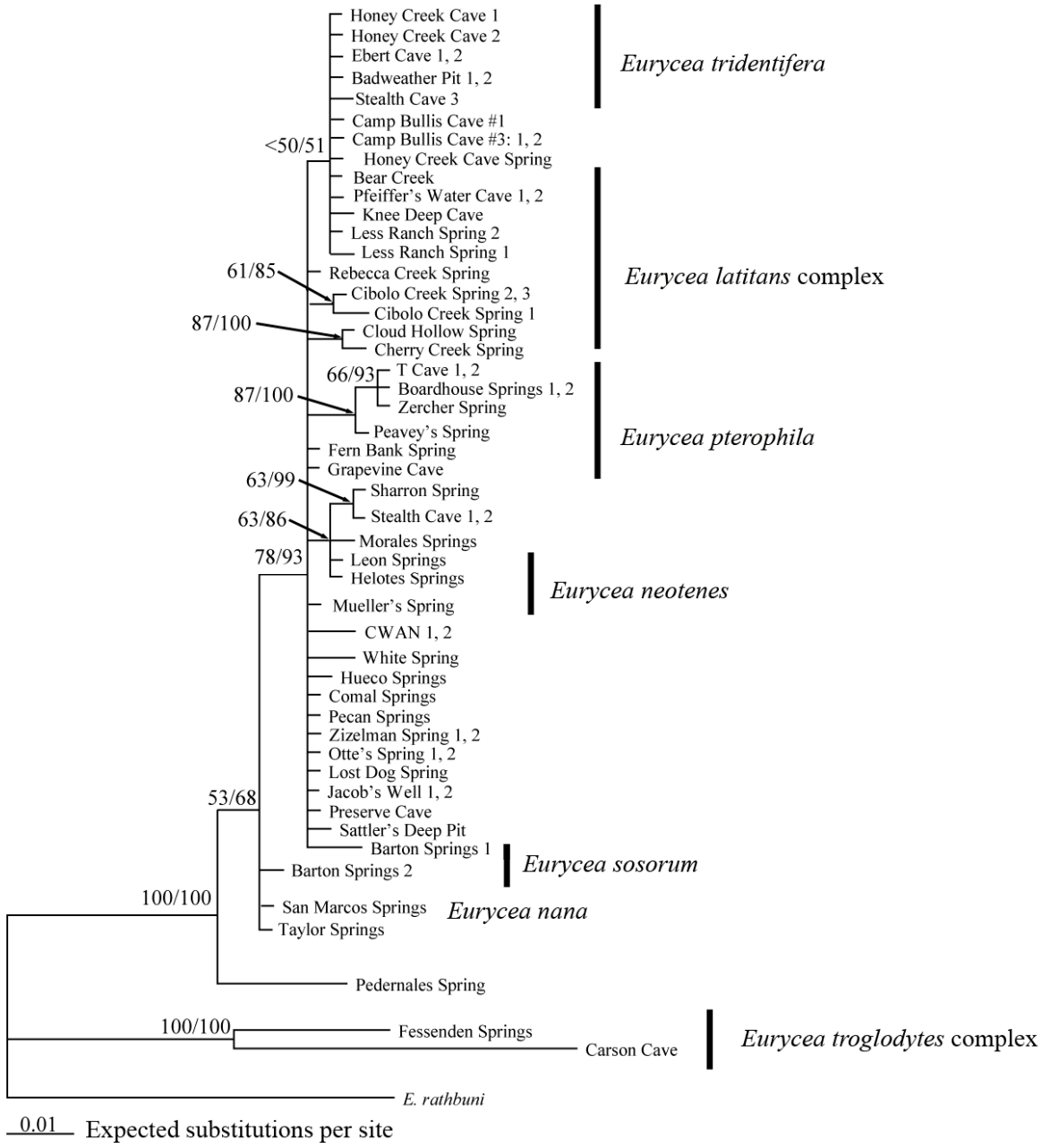

Figure 3.4. Bayesian ND2 phylogram. The Bayesian topology was similar to the parsimony topology, and bootstrap values are mapped to the Bayesian phylogram. Numbers at nodes represent parsimony bootstrap values followed by Bayesian posterior probabilities (MP/PP). Species names are shown for populations assigned to species by Chippindale et al. (2000).

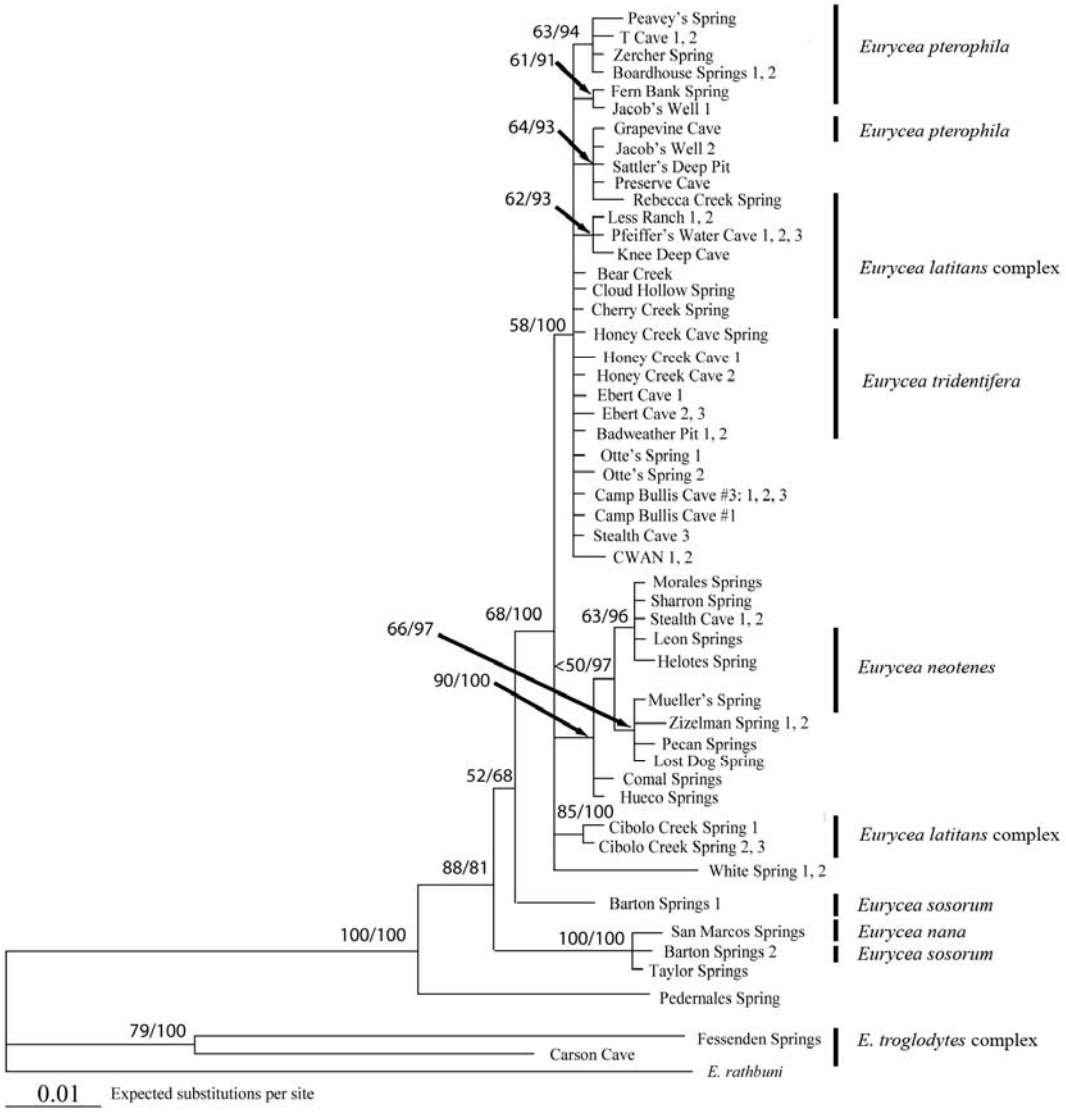

Figure 3.5. Bayesian Cyt*b* phylogram. The Bayesian topology was similar to the parsimony topology, and bootstrap values are mapped to the Bayesian phylogram. Numbers at nodes represent parsimony bootstrap values followed by Bayesian posterior probabilities (MP/PP). Species names are shown for populations assigned to species by Chippindale et al. (2000).

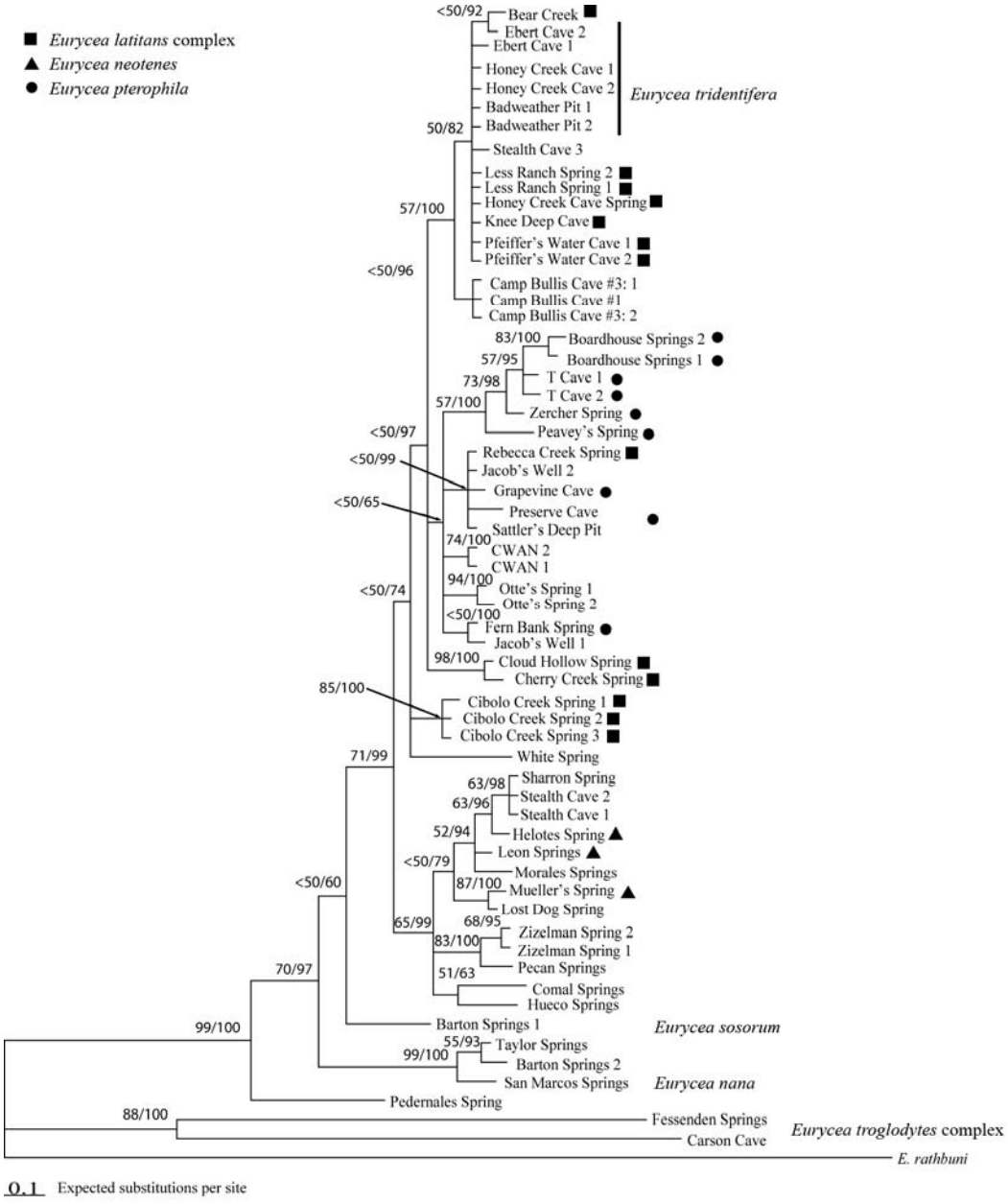

Figure 3.6. Bayesian IGS/CR phylogram. The Bayesian topology was more resolved than the parsimony topology, but no nodes were in conflict, and bootstrap values are mapped to the Bayesian phylogram. Numbers at nodes represent parsimony bootstrap values followed by Bayesian posterior probabilities (MP/PP). Species names are shown for populations assigned to species by Chippindale et al. (2000).

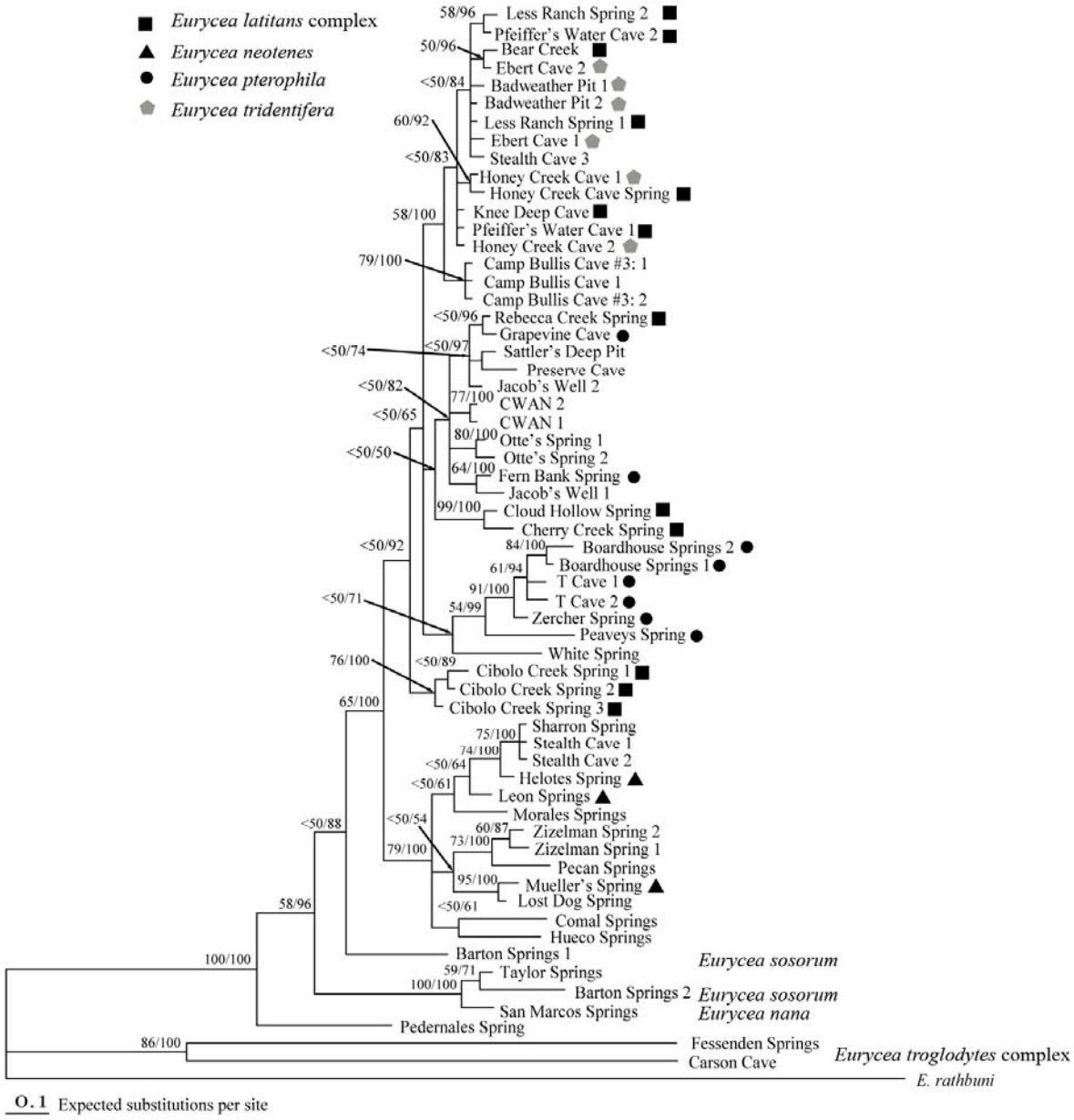

Figure 3.7. Bayesian IGS/CR+gaps phylogram. The Bayesian topology was more resolved than the parsimony topology, but no nodes were in conflict, and bootstrap values are mapped to the Bayesian phylogram. Numbers at nodes represent parsimony bootstrap values followed by Bayesian posterior probabilities (MP/PP). Species names are shown for populations assigned to species by Chippindale et al. (2000).

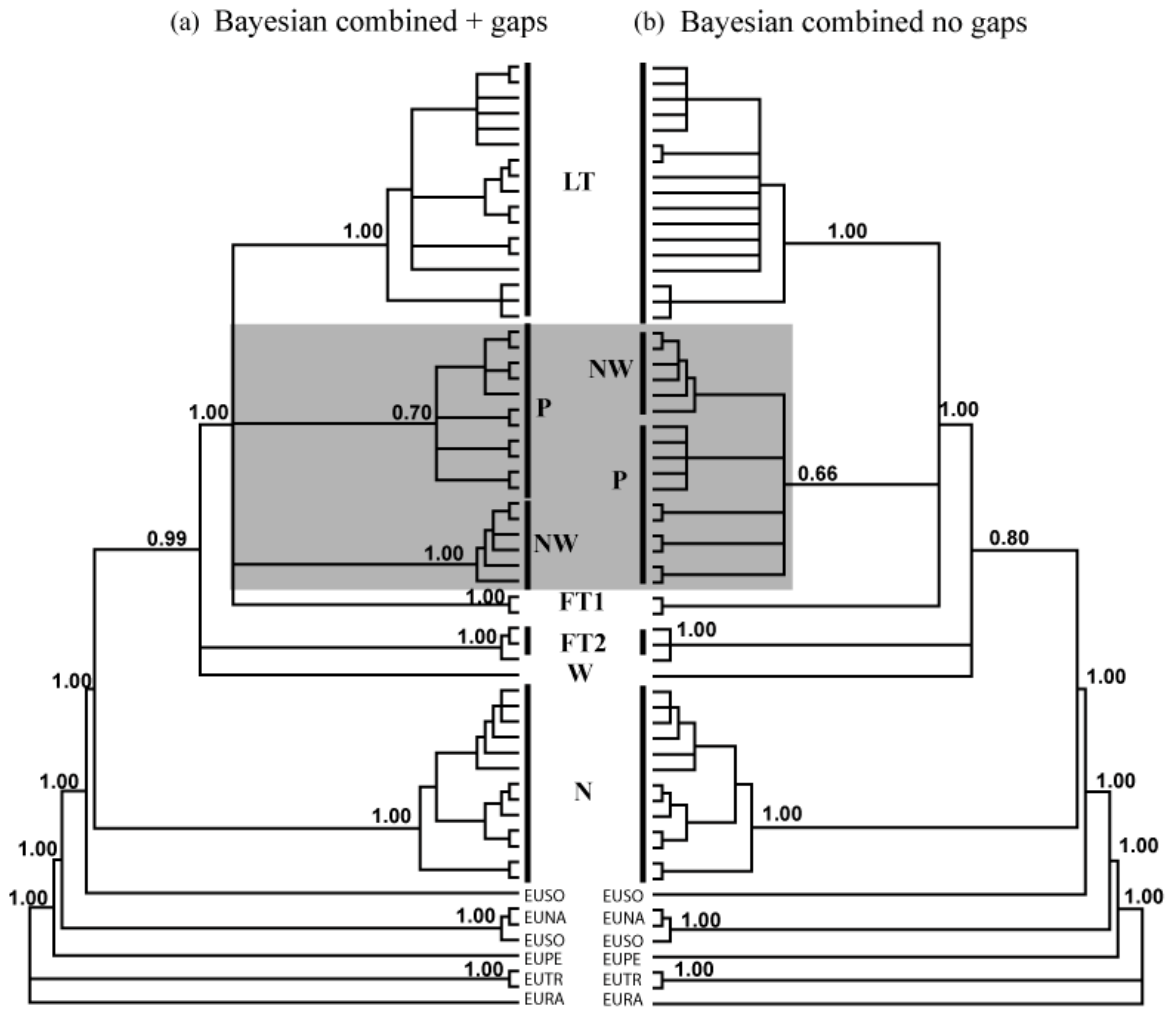

Figure 3.8. Cladogram from combined Bayesian analysis including (a) and excluding (b) gap characters from the IGS/CR partition. Support values are shown only for selected nodes. Areas of the tree where topologies differed between gap inclusion and exclusion are highlighted in grey. **N**, *E. neotenes* and closely related populations; **W**, population at White Spring; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **SE**, clade of *E. pterophila* and closely related populations with a South/East distribution; **NW**, clade of *E. pterophila* and closely related populations with a North/West distribution; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E. pterophila* and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P.

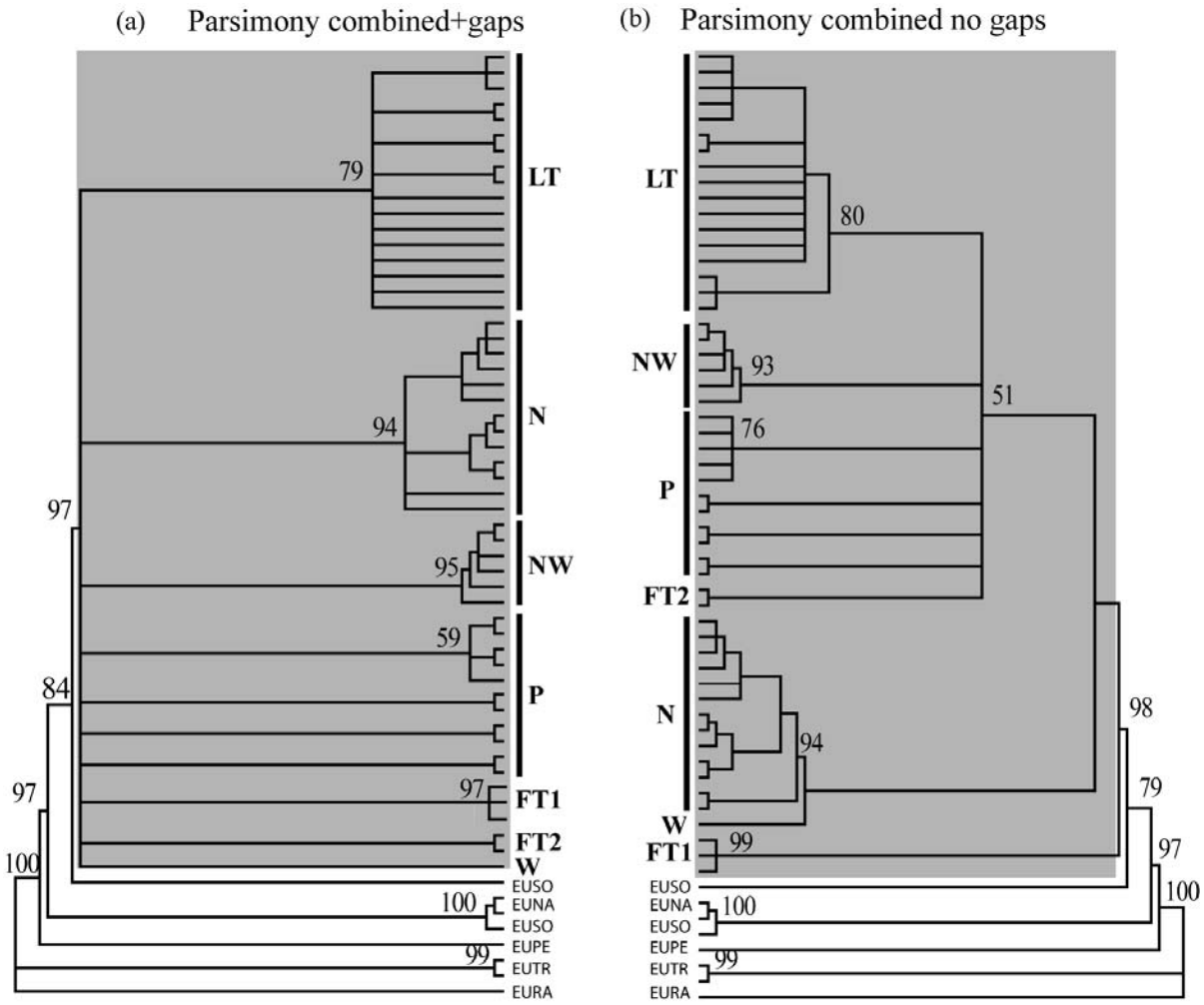

Figure 3.9. Cladogram from combined parsimony analysis including (a) and excluding (b) gap characters from the IGS/CR partition. Support values are shown only for selected nodes. Areas of the tree where topologies differed between gap inclusion and exclusion are highlighted in grey. **N**, *E. neotenes* and closely related populations; **W**, population at White Spring; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **SE**, clade of *E. pterophila* and closely related populations with a South/East distribution; **NW**, clade of *E. pterophila* and closely related populations with a North/West distribution; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E. pterophila* and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P.

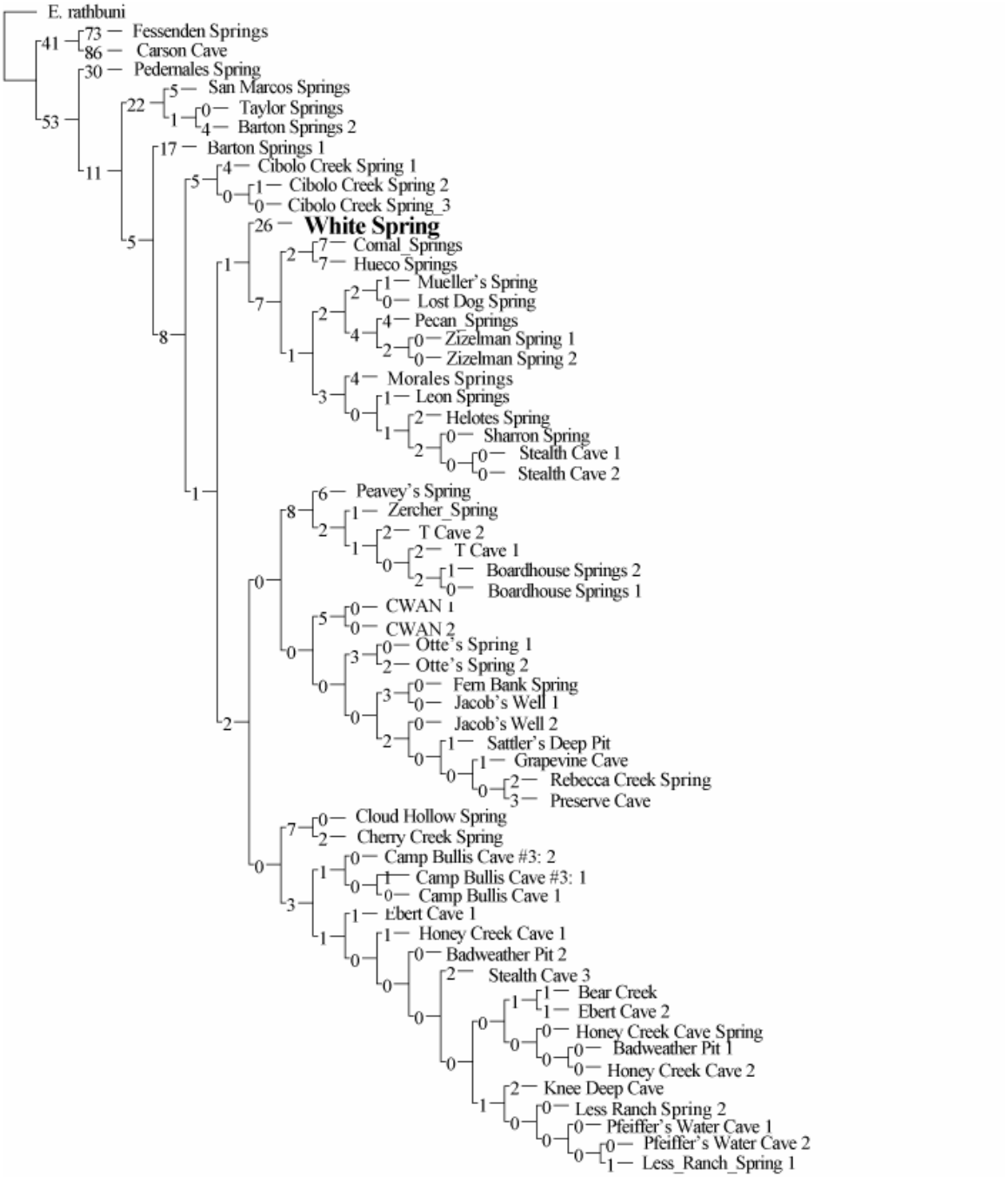

Figure 3.10. One of three equally parsimonious trees of the combined1 dataset. Number of steps for each branch are mapped to the tree. White Spring, highlighted in bold, has an unusually long branch of 26 steps.

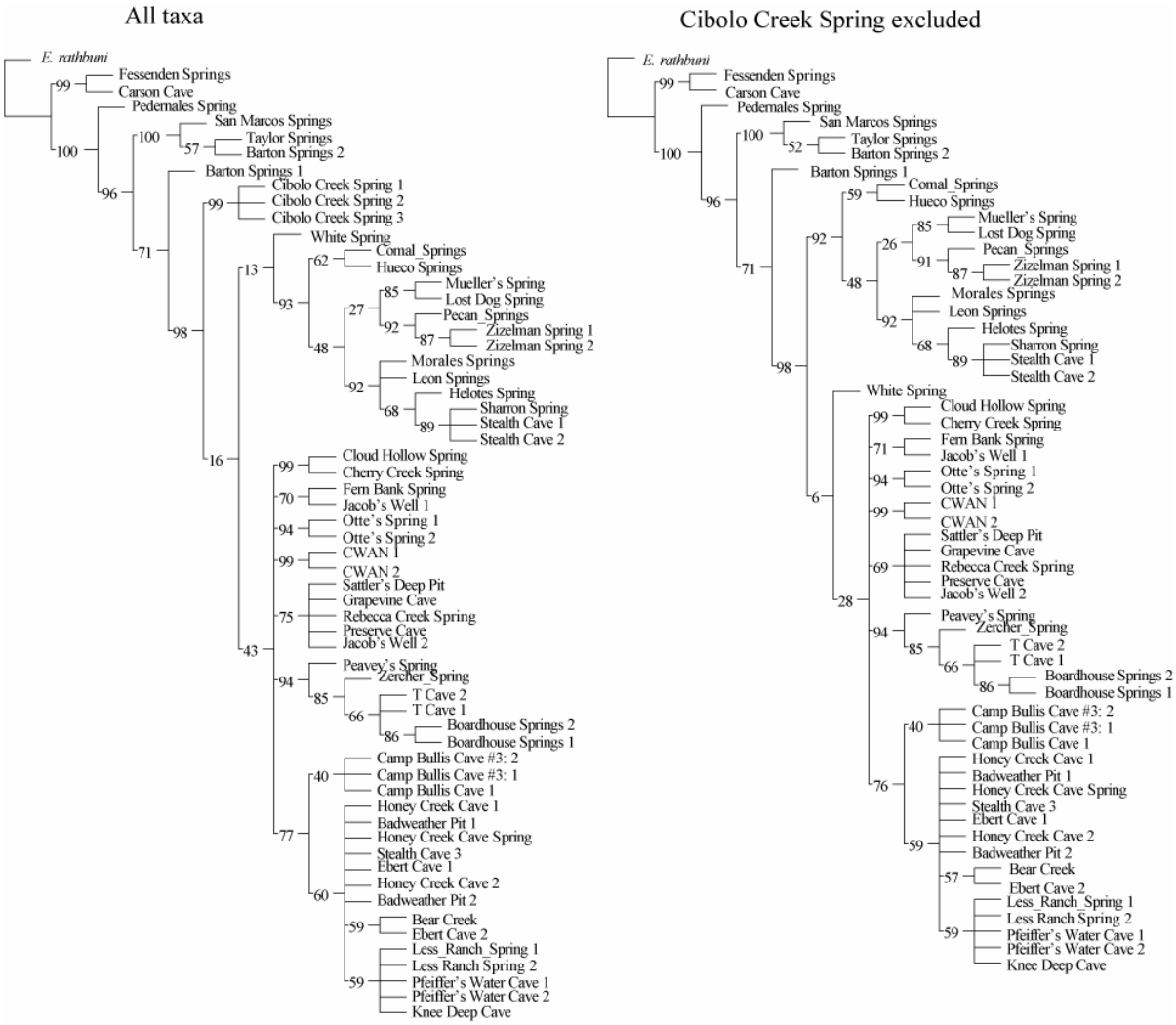

Figure 3.11. Parsimony cladograms with and without Cibolo Creek Spring. Node support is displayed as GC values (difference between most supported and next most supported contradictory groups [Goloboff et al. 2003a]). Node support decreased but the position of White Spring changed.

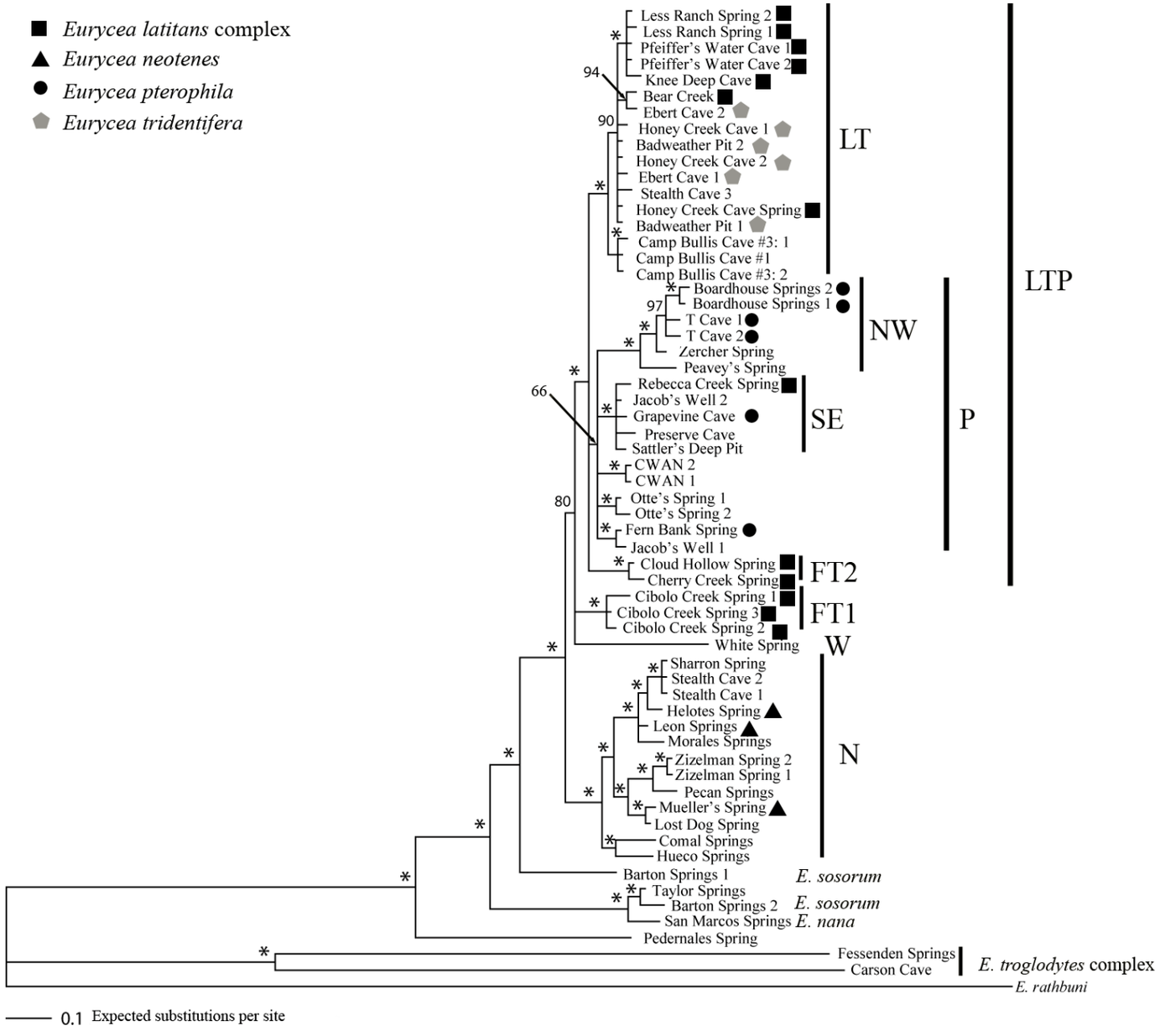

Figure 3.12. Phylogram resulting from the Bayesian analysis of combined1 dataset. **N**, *E. neotenes* and closely related populations; **W**, population at White Spring; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **SE**, clade of *E. pterophila* and closely related populations with a South/East distribution; **NW**, clade of *E. pterophila* and closely related populations with a North/West distribution; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E. pterophila* and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P. Species names are shown for populations assigned to species by Chippindale et al. (2000).

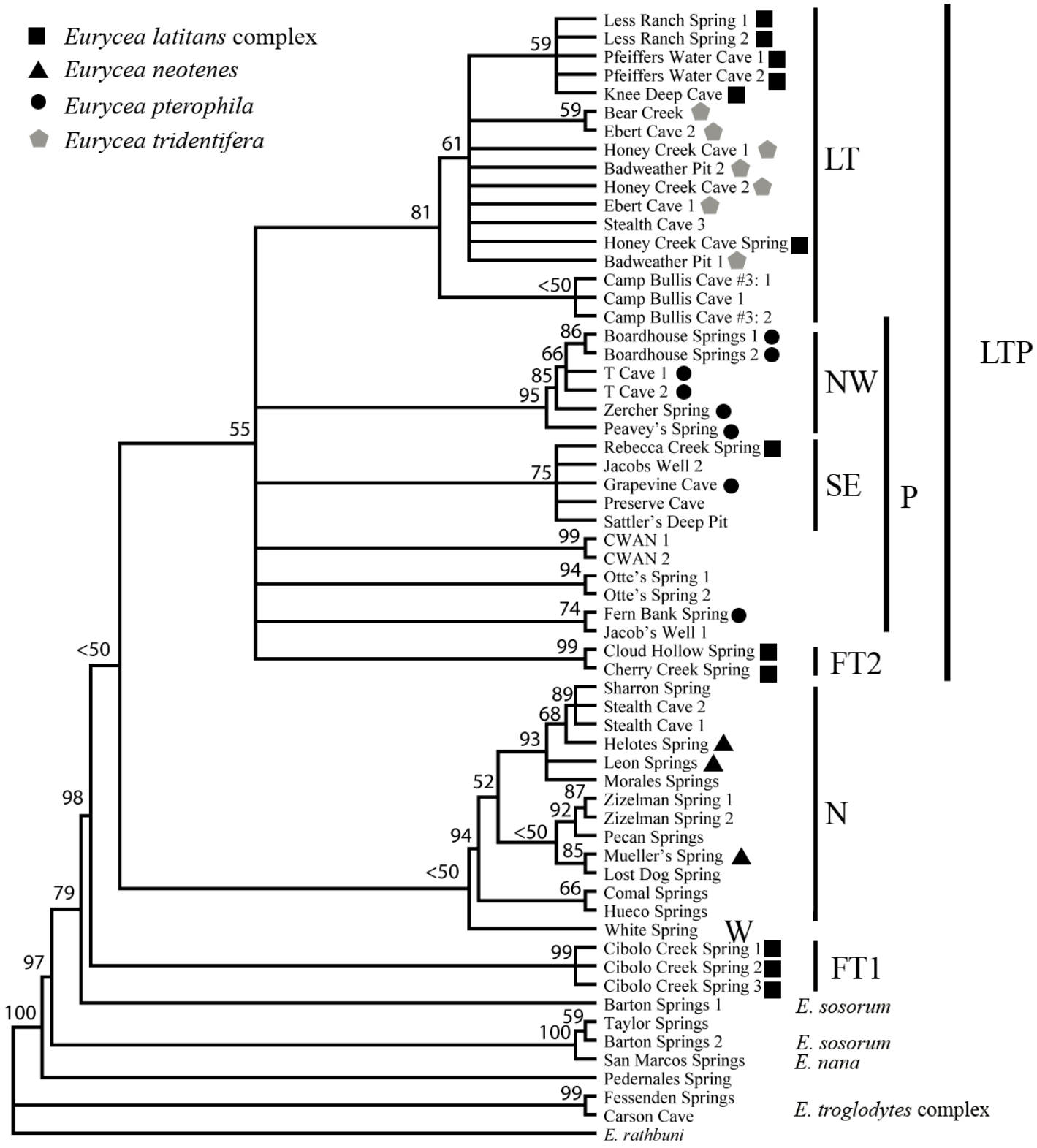

Figure 3.13. Parsimony cladogram of combined1 dataset resulting from the strict consensus of three equally parsimonious trees. Bootstrap values are displayed above nodes. **N**, *E. neotenes* and closely related populations; **W**, population at White Spring; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **SE**, clade of *E. pterophila* and closely related populations with a South/East distribution; **NW**, clade of *E. pterophila* and closely related populations with a North/West distribution; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E. pterophila* and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P. Species names are shown for populations assigned to species by Chippindale et al. (2000).

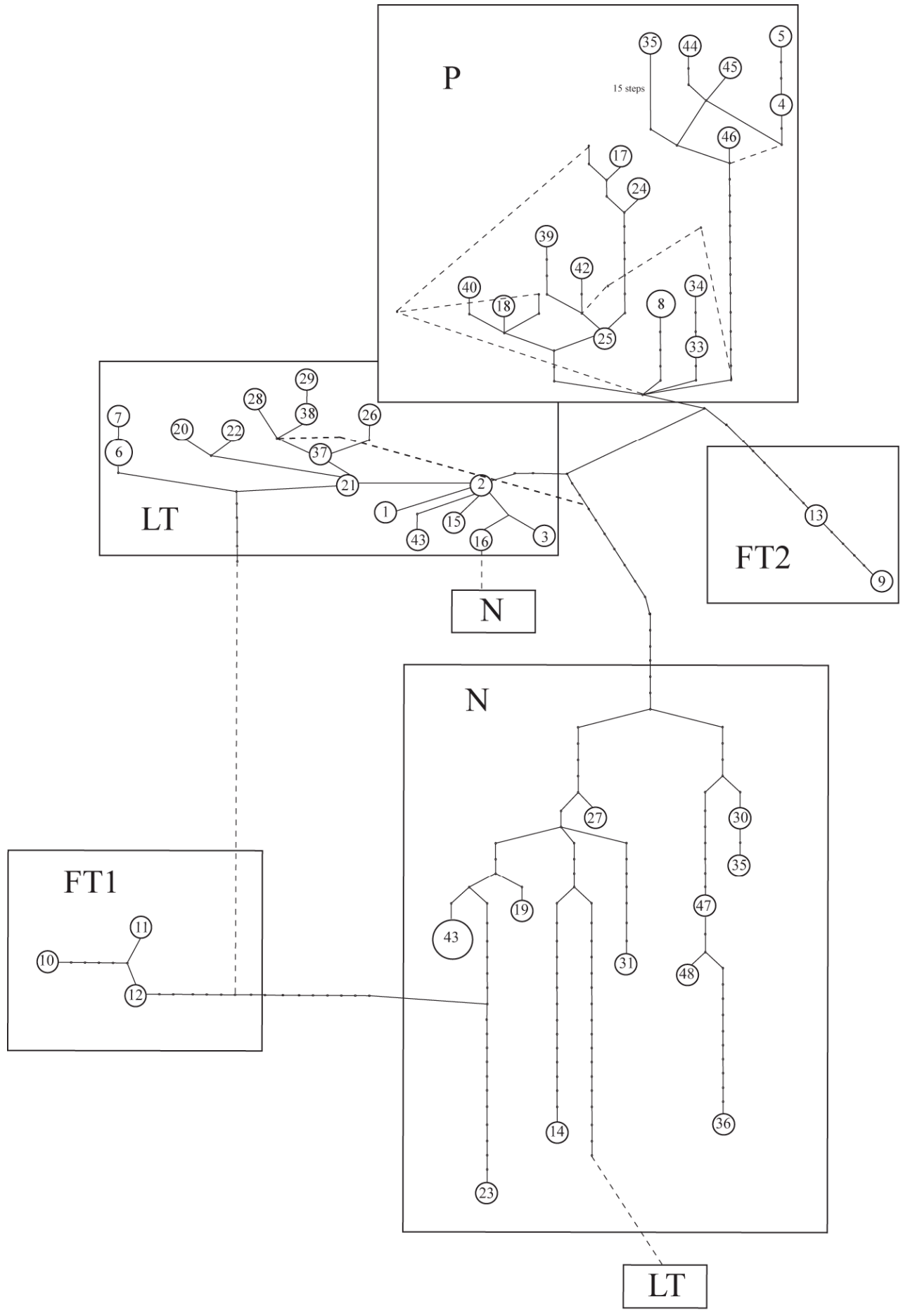

Figure 3.14. Haplotype network of clades N, LT, P, FT1, and FT2. Loops are indicated as dashed lines. Each segment equals one step and small dots indicate haplotypes not present in sampling. Haplotype designations are shown in circles and defined in table 5; circle size indicates relative frequency of haplotype. **N**, *E. neotenes* and closely related populations; **FT1**, clade 1 of populations within the Fort Terrett formation; **FT2**, clade 2 of populations within the Fort Terrett formation; **LT**, *E. latitans*, *E. tridentifera*, and other closely related populations; **P**, group inclusive of all *E. pterophila* and related or geographically proximate populations; **LTP**, *E. latitans*, *E. tridentifera*, *E. pterophila* and related populations, inclusive of clades FT2, LT and P.

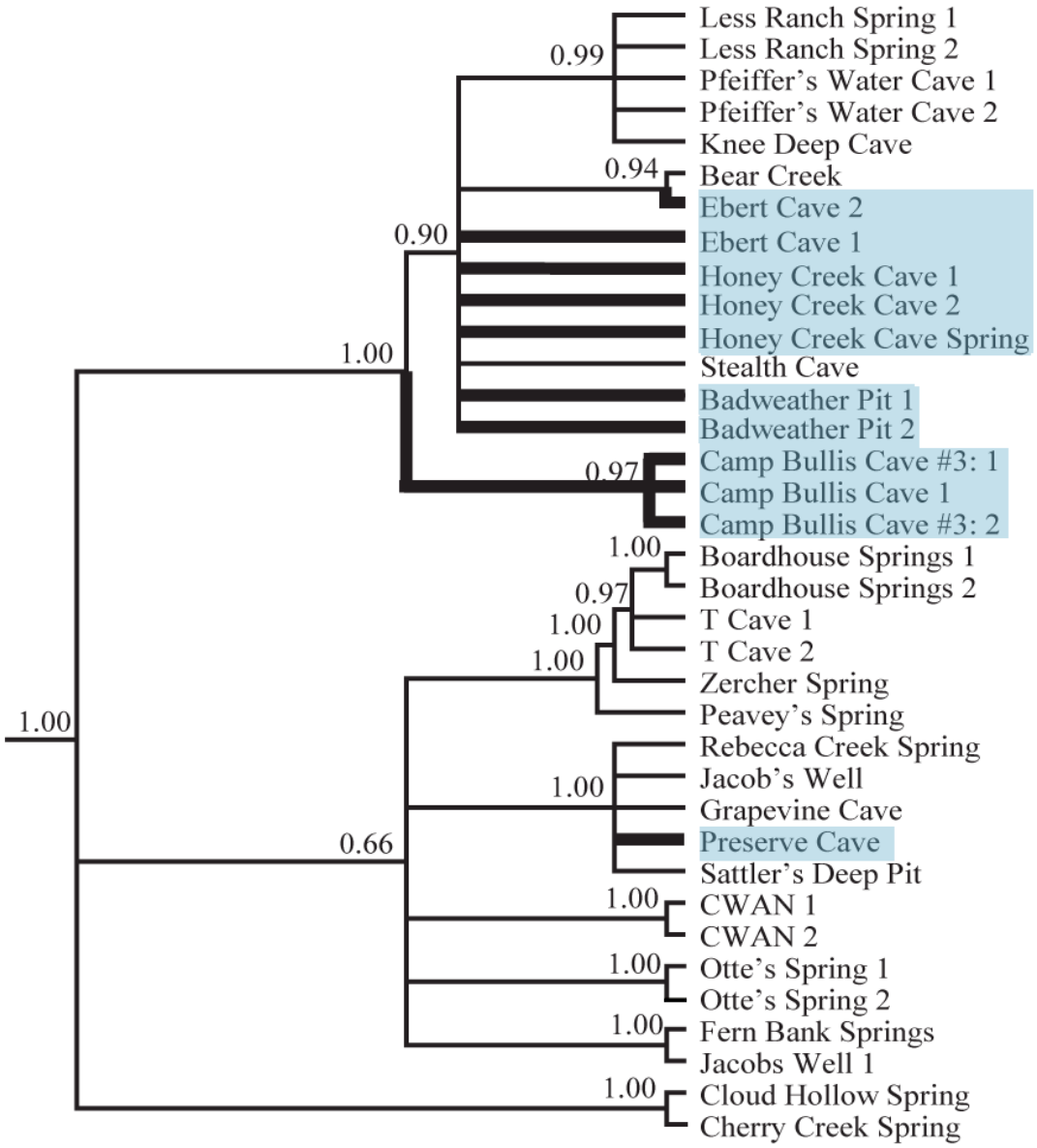

Figure 3.15. Portion of Bayesian cladogram depicting relationships among troglomorphic and non-troglomorphic taxa (branches indicated are in bold and taxa are highlighted for troglomorphic specimens). The phylogenetic positions of Preserve Cave, and of troglomorphic taxa sister to Camp Bullis Cave, indicate possible convergent evolution of cave-associated features.

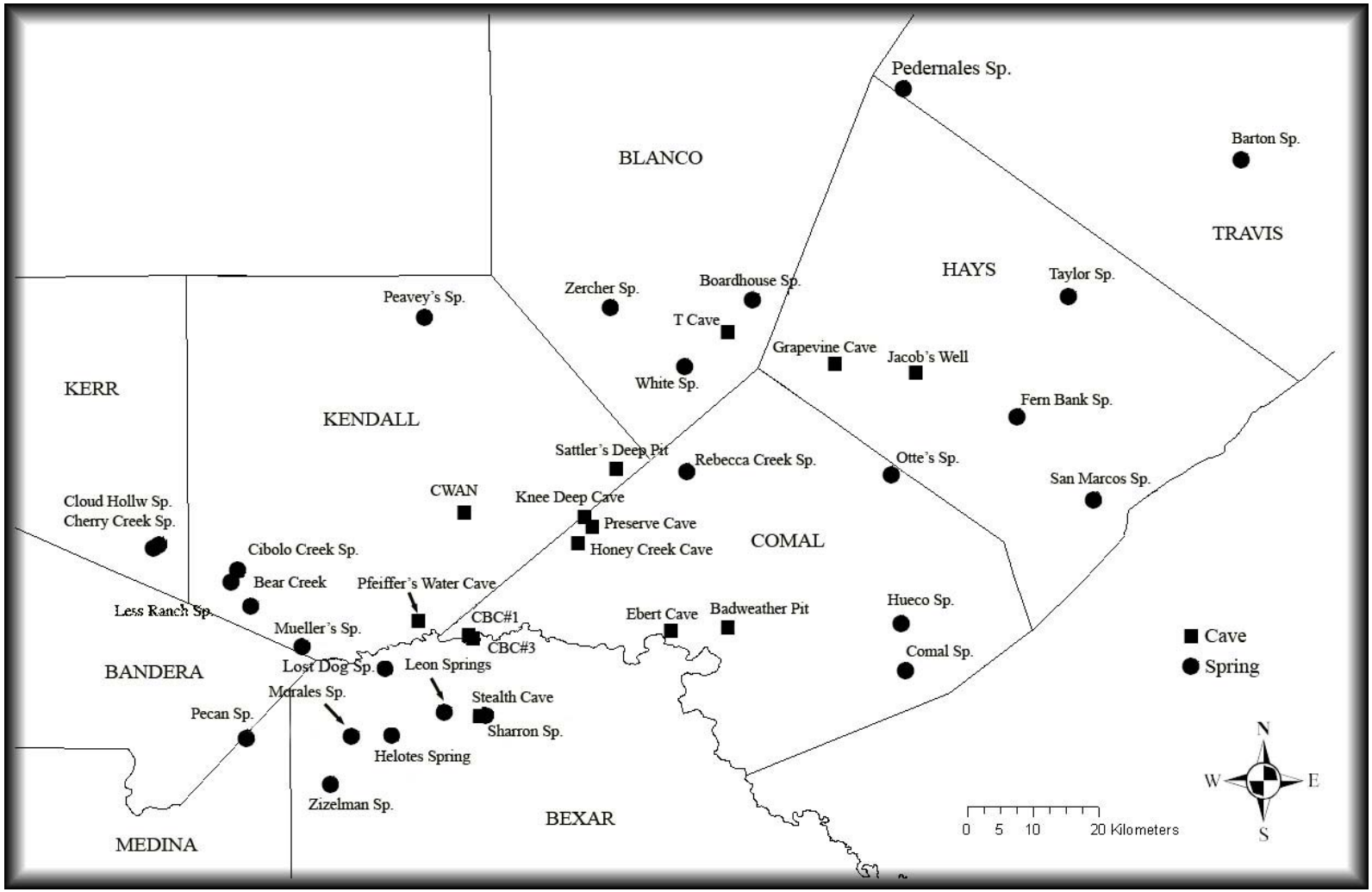

Figure 3.16. Distribution of *Eurycea* included in this study. Caves are indicated as squares, springs as circles.

Honey Creek Cave

Preserve Cave

Camp Bullis Cave 3

Figure 3.17. Photographs of topotypical *E. tridentifera* from Honey Creek Cave and salamanders from localities previously unassigned to species, Preserve Cave and Camp Bullis Cave 3. Photographs are not to scale.

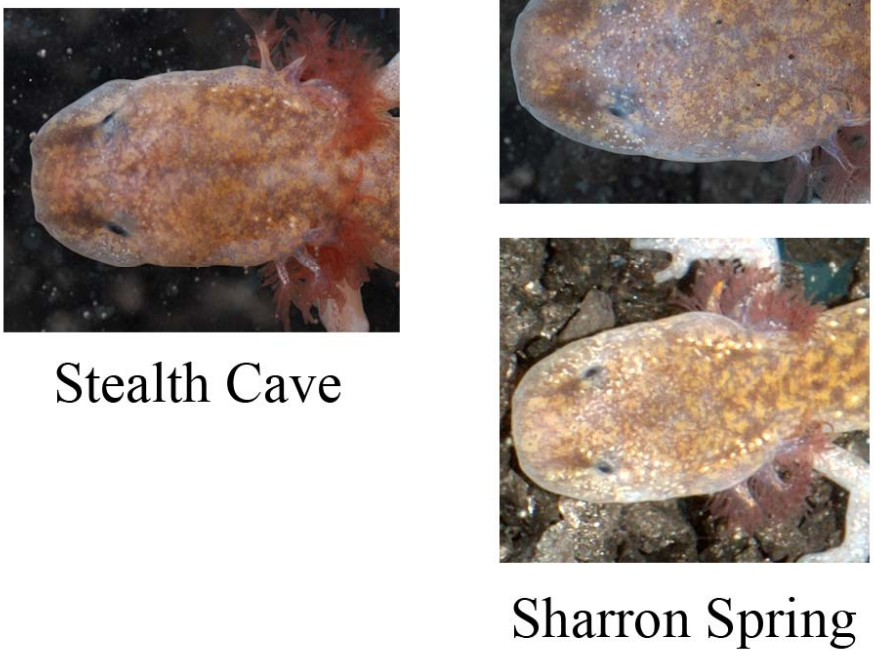

Figure 3.18. Head shapes of Stealth Cave specimen 3 and two Sharron Spring salamanders.

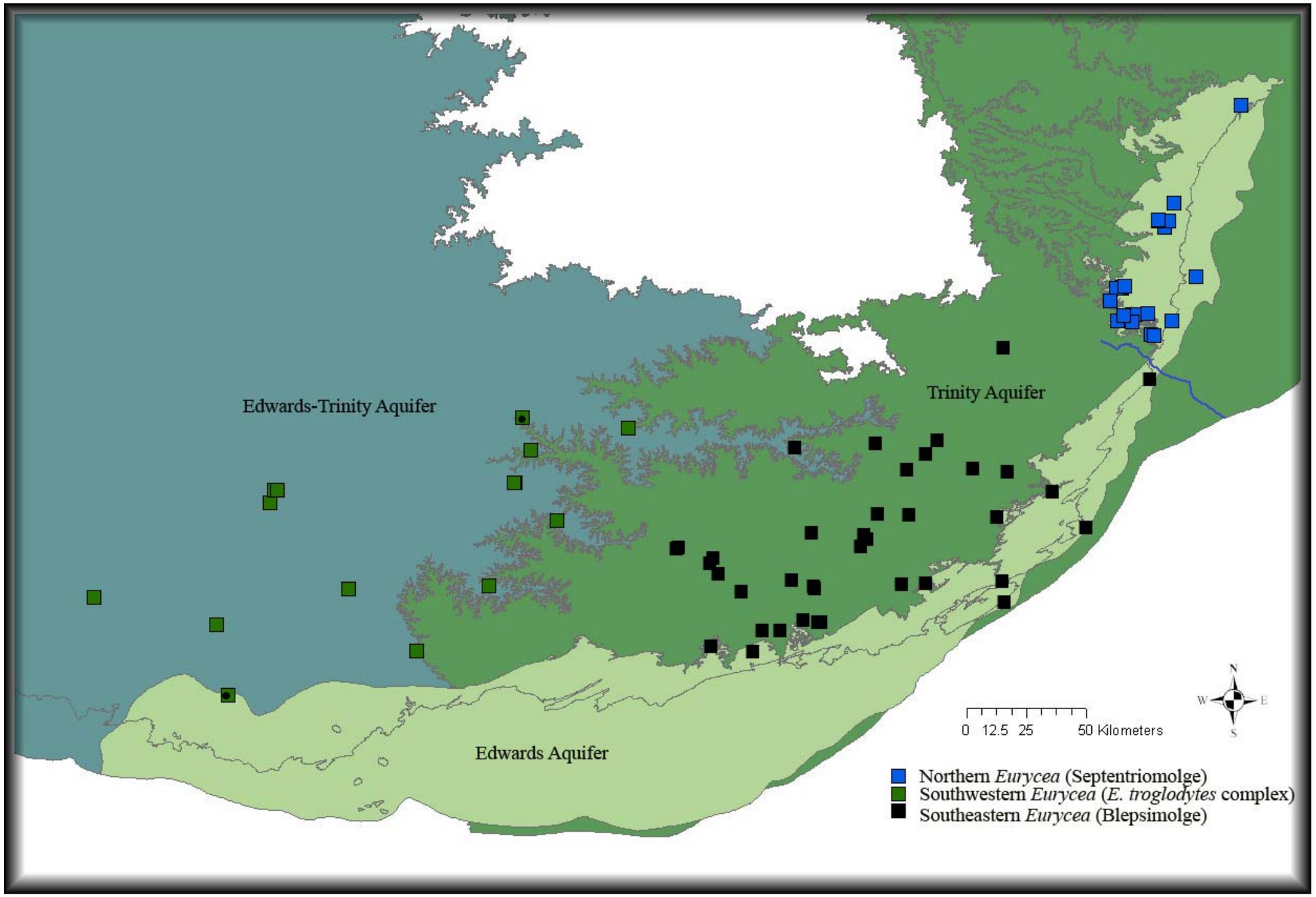

Figure 3.19. Major aquifer boundaries of the Edwards Plateau and sampling localities of specimens from molecular studies. Black squares represent sampling localities for southeastern Blepsimolge included in this study. Green and blue squares are sampling localities for southwestern Blepsimolge and Septentriomolge, respectively, from Chippindale et al. (2000). Green squares with black circles indicate the two *E. troglodytes* populations included in this study.

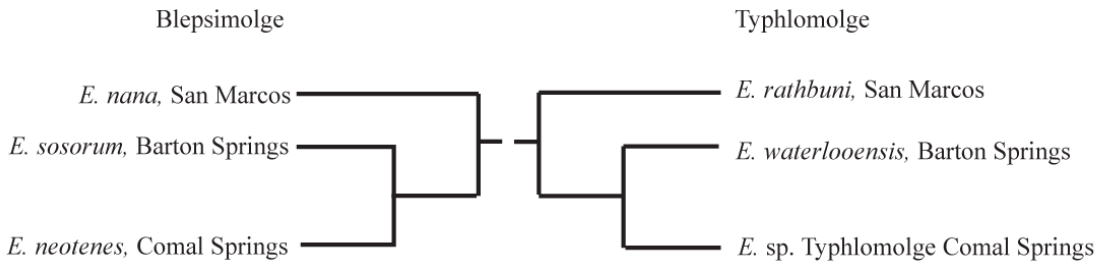

Figure 3.20. Relationships among Blepsimolge and Typhlomolge at San Marcos, Barton Springs and Comal Springs (unpublished data; other OTUs excluded).

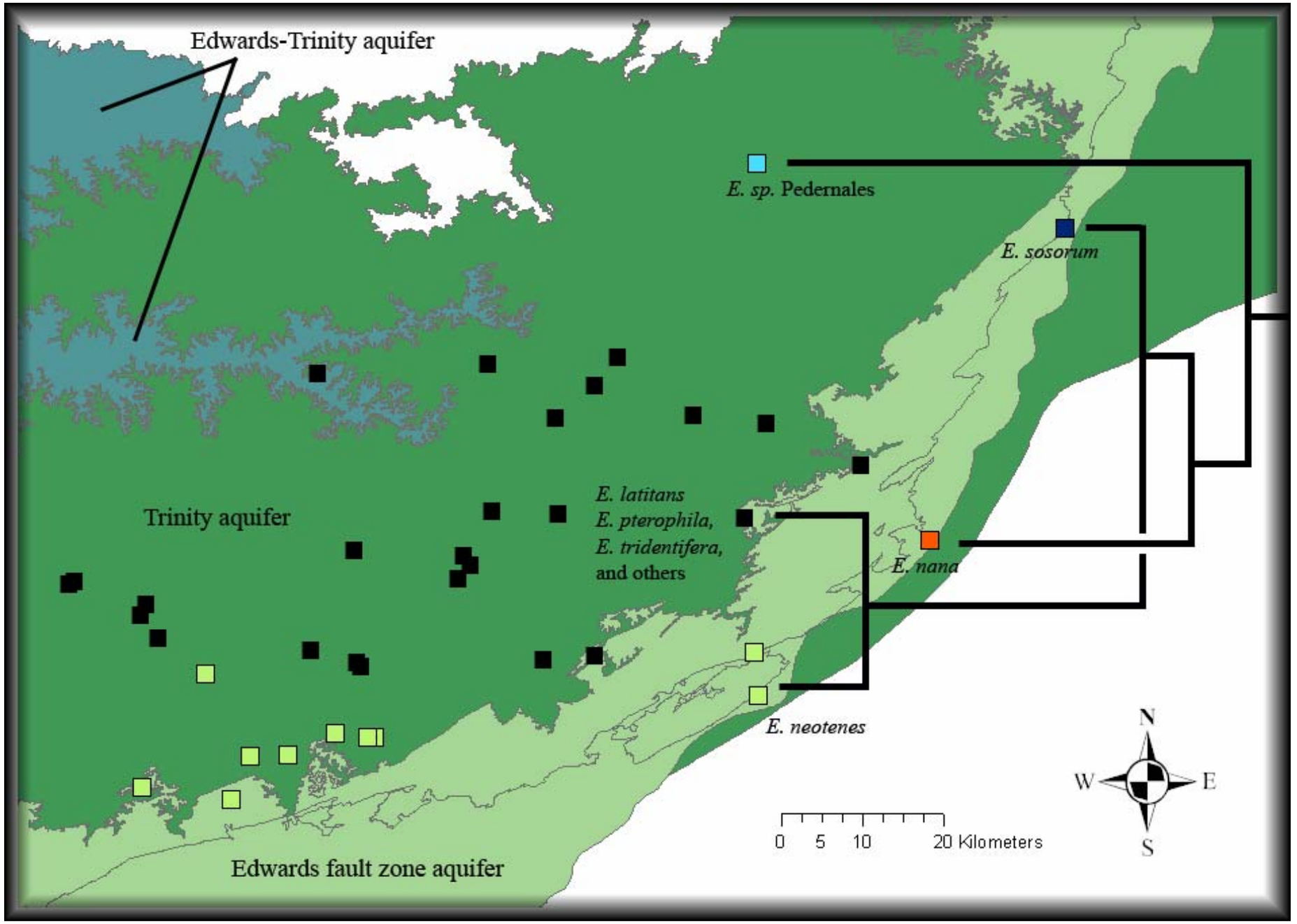

Figure 3.21. Relationships among the major groups of the southeastern Blepsimolge and their distribution with respect to the major aquifers of the Edwards Plateau. Divergences among the taxa inhabiting the peripheral range of the southeastern Blepsimolge (particularly within the Edwards fault zone) are the oldest. One possible explanation of this pattern is that past speciation events may have occurred due to compartmentalization within the Edwards Aquifer, leading to the current state where there are no known hydrological connections among the largest springs (Comal & Hueco, San Marcos, and Barton Springs).

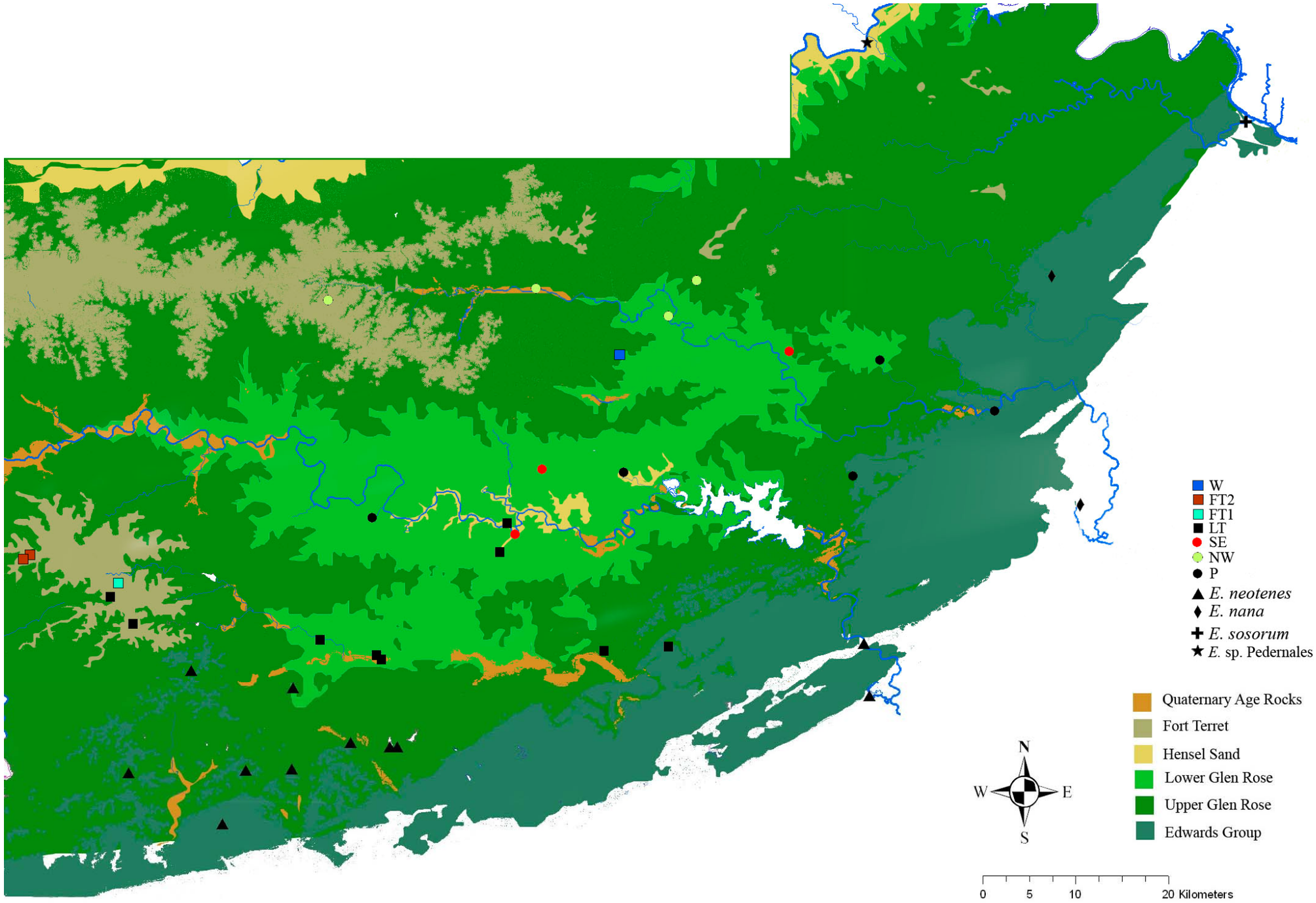

Figure 3.22. Distribution of southeastern Blepsimolge with respect to the major exposed geologic formations of the Edwards Plateau.

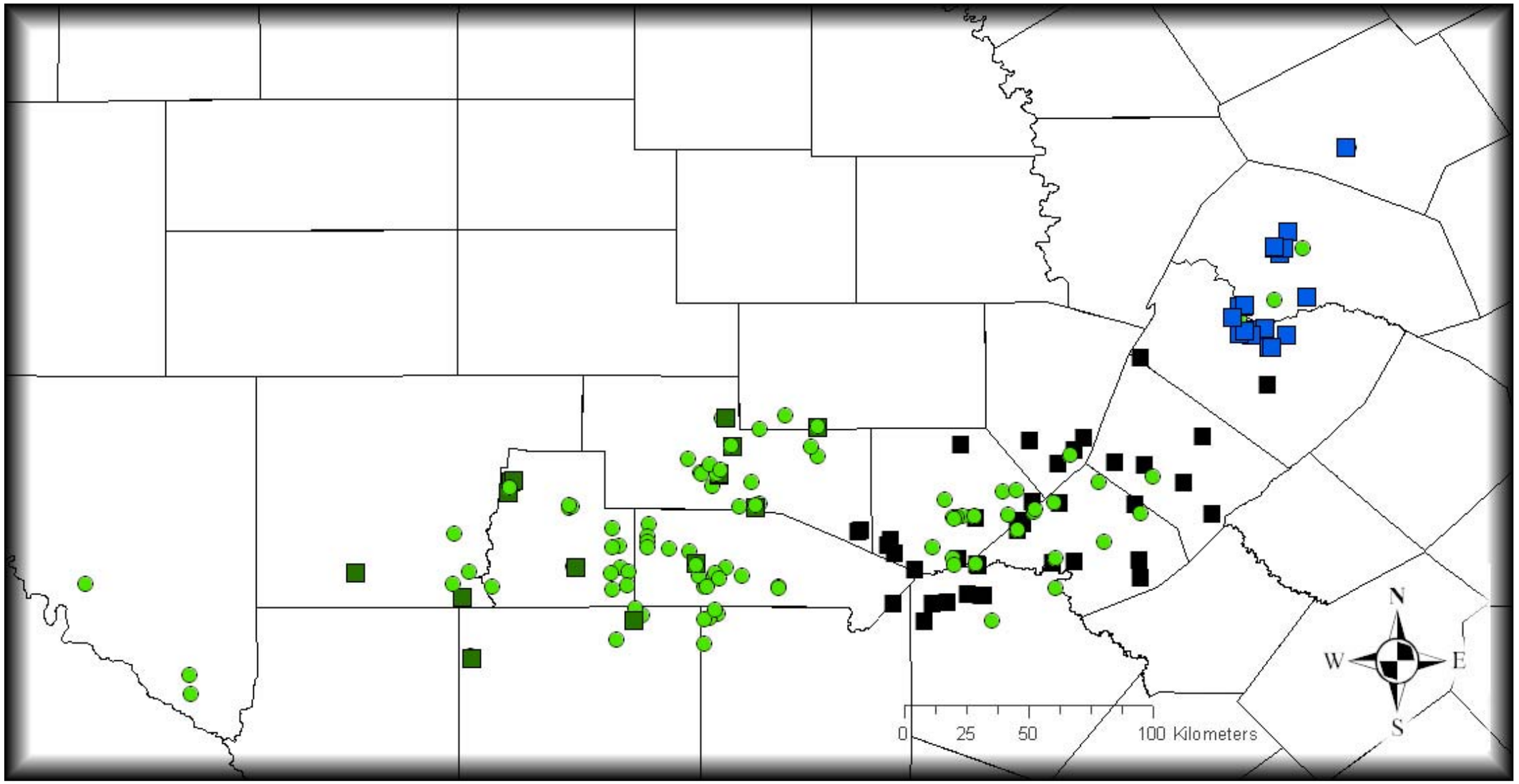

Figure 3.23. Known localities of *Eurycea* in the Edwards Plateau. Squares represent localities represented in molecular studies: black= southeastern Blepsimolge; blue= Septentriomolge, Chippindale et al. (2000); green= southwestern Blepsimolge, Chippindale et al. (2000); Circles represent other known localities from Sweet (1978a) and other sources.

APPENDIX A

LOCALITIES AND MUSEUM NUMBERS FOR SPECIMENS USED

Locality	Field Number	Museum Number	Latitude	Longitude
Badweather Pit 1	DMH 90-113	n/a	29.75606	-98.32974
Badweather Pit 2	DMH 90-117	TNHC 64499	29.75606	-98.32974
Barton Springs 1	DMH 88-141	n/a	30.26382	-97.77084
Barton Springs 2	DMH/PC 92-109	TNHC 51181	30.26382	-97.77084
Bear Creek	DMH 90-123	n/a	29.80439	-98.86975
Boardhouse Springs 1	DMH 88-22	n/a	30.11132	-98.30224
Boardhouse Springs 2	DMH 88-31	n/a	30.11132	-98.30224
Camp Bullis Cave #1	AGG 827	UTA A	29.74777	-98.61179
Camp Bullis Cave #3 1	AGG 823	UTA A 56722	n/a	
Camp Bullis Cave #3 2	AGG 825	UTA A 56719	n/a	
Camp Bullis Cave #3 3	AGG 826	UTA A 56721	n/a	
Carson Cave	DMH 90-74	n/a	29.47476	-100.07363
Cave Without A Name 1	AGG 1058	UTA A 57289	29.88096	-98.61613
Cave Without A Name 2	AGG 1059	UTA A 57290	29.88096	-98.61613
Cherry Creek Spring	DMH 92-66	TNHC 54531	29.84494	-98.94809
Cibolo Creek Spring 1	DMH 90-131	n/a	29.81772	-98.86225
Cibolo Creek Spring 2	DMH 90-129	n/a	29.81772	-98.86225
Cibolo Creek Spring 3	DMH 90-130	n/a	29.81772	-98.86225
Cibolo Creek Spring 4	DMH 90-132	n/a	29.81772	-98.86225
Cibolo Creek Spring 5	DMH 90-125	n/a	29.81772	-98.86225
Cloud Hollow Spring	DMH/PC 92-86	TNHC 59934	29.84078	-98.95420
Comal Springs	DMH 90-177	n/a	29.70817	-98.13550
Comal Springs	NFB 012	n/a	29.70817	-98.13550
Comal Springs	NFB 013	n/a	29.70817	-98.13550
Comal Springs	NFB 014	n/a	29.70817	-98.13550
Comal Springs	DMH 90-175	n/a	29.70817	-98.13550
Comal Springs	AHP 3081	n/a	29.70817	-98.13550
Comal Springs	RMB 2941	n/a	29.70817	-98.13550
Comal Springs	RMB 2942	n/a	29.70817	-98.13550
Comal Springs	RMB 2943	n/a	29.70817	-98.13550
E. rathbuni, Rattlesnake Cave	DMH 91-10	TNHC 51175	n/a	
Ebert Cave 1	DMH/PC 92-26	n/a	29.75189	-98.39140
Ebert Cave 2	PC/DMH 92-100	TNHC 64502	29.75189	-98.39140
Ebert Cave 3	DMH/PC 93-28	TNHC 54542	29.75189	-98.39140
Fern Bank Spring 1	DMH 89-02	n/a	29.98355	-98.01390
Fern Bank Spring 2	DMH 89-04	n/a	29.98355	-98.01390
Fessenden Springs	AGG 1033	UTA A 57265	30.16645	-99.33832
Grapevine Cave	PC/DMH 92-50	TNHC 51113	30.04188	-98.21279
Helotes Spring 1	DMH 90-158	n/a	29.63773	-98.69475
Helotes Spring 2	DMH 90-138	n/a	29.63773	-98.69475
Honey Creek Cave 1	DMH 91-57	TNHC 51057	29.84744	-98.49196
Honey Creek Cave 2	DMH/PC 93-62	TNHC 53856	29.84744	-98.49196
Honey Creek Cave Spring	DMH/PC 91-58	TNHC 51166	29.84744	-98.49196
Hueco Springs	NFB 010	n/a	29.75967	-98.14056
Jacob's Well 1	ZARA 381	n/a	30.03300	-98.12550

Jacob's Well 2	JKK 2003-1/NFB009	UTA A 57249	30.03300	-98.12550
Knee Deep Water Cave	AGG 1032	UTA A 57264	29.87549	-98.48502
Leon Springs 1	DMH 90-136	n/a	29.66300	-98.63752
Leon Springs 2	DMH/PC 92-6	n/a	29.66300	-98.63752
Less Ranch Spring 1	DMH/PC 93-65	n/a	29.77800	-98.84808
Less Ranch Spring 2	DMH/PC 93-66	TNHC 64504	29.77800	-98.84808
Lost Dog Spring	AGG 1040	UTA A 57271	29.70647	-98.69068
Morales Springs	AGG 1065	UTA A 57279	29.63661	-98.73921
Mueller's Spring	DMH 90-233	n/a	29.73356	-98.79197
Otte's Spring 1	AGG 1041	UTA A 57272	29.92051	-98.15167
Otte's Spring 2	AGG 1042	UTA A 57273	29.92051	-98.15167
Peavey's Springs	DMH 90-279	n/a	30.09188	-98.65864
Pecan Springs	DMH 94-4	TNHC 55128	29.63417	-98.85269
Pedernales Spring #1	DMH 91-56A	TNHC 51048	30.34131	-98.13752
Pfeiffer's Water Cave 1	DMH/PC 92-102	TNHC 54534	29.76244	-98.66669
Pfeiffer's Water Cave 2	DMH/PC 92-104	TNHC 54536	29.76244	-98.66669
Pfeiffer's Water Cave 3	DMH/PC 92-106	TNHC 54538	29.76244	-98.66669
Preserve Cave	AGG 1026	n/a	29.86458	-98.47750
Rebecca Springs	DMH 89-18	n/a	29.92466	-98.37307
San Marcos Springs	PC 1997-11	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-12	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-13	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-14	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-15	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-16	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-17	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-19	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-21	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-22	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-23	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-24	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-25	n/a	29.89327	-97.93084
San Marcos Springs	PC 1997-26	n/a	29.89327	-97.93084
San Marcos Springs	1	MF 17848	29.89327	-97.93084
San Marcos Springs	2	MF 17849	29.89327	-97.93084
San Marcos Springs	3	MF 17850	29.89327	-97.93084
San Marcos Springs	4	MF 17851	29.89327	-97.93084
San Marcos Springs	6	MF 17854	29.89327	-97.93084
San Marcos Springs	7	MF 17855	29.89327	-97.93084
San Marcos Springs	8	MF 17856	29.89327	-97.93084
San Marcos Springs	DMH 88-208	TNHC 52758	29.89327	-97.93084
San Marcos Springs	DMH 88-202	n/a	29.89327	-97.93084
San Marcos Springs	DMH 90-182	n/a	29.89327	-97.93084
Sattler's Deep Pit	PC 94-02	TNHC 54020	29.92820	-98.45104
Sharron Spring 1	AGG 1014	UTA A 57299	n/a	
Sharron Spring 2	AGG 832	UTA A 57276	n/a	
Stealth Cave 1	AGG 1030	UTA A 57262	n/a	

Stealth Cave 2	AGG 1031	UTA A 57263	n/a	
Stealth Cave 3	AGG 1029	UTA A 57261	n/a	
T Cave 1	DMH 90-201	TNHC 51116	30.07688	-98.32974
T Cave 2	DMH 90-265	TNHC 51118	30.07688	-98.32974
Taylor Springs	n/a	n/a	30.11475	-97.95858
White Spring 1	AGG 1011	UTA A 56723	30.03920	-98.37655
White Spring 2	AGG 1060	UTA A 57291	30.03920	-98.37655
Zercher Spring	DMH 90-203	TNHC 64488	30.10299	-98.45724
Zizelman Spring 1	AGG 1036	UTA A 57267	29.58465	-98.76155
Zizelman Spring 2	AHP 3367	TNHC 57742	29.58465	-98.76155

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BIOGRAPHICAL INFORMATION

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