FINAL REPORT

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# Project No. 3.4: Status Report of Central Texas Salamanders (Genus: *Eurycea*)


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**FINAL REPORT**

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**OBJECTIVE:** To investigate the taxonomic status, distribution and relative abundance, habitat requirements, and nature and degree of potential threats to populations of endemic central Texas salamanders belonging to *Eurycea* and *Typhlomolge* genera (Family: Plethodontidae).

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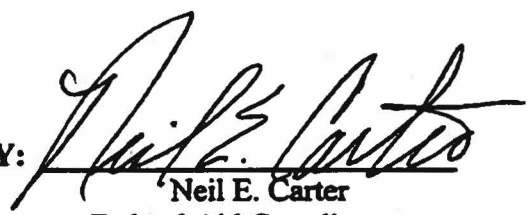
**APPROVED BY:**  **DATE:** 2-4-2000

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## Executive Summary

The purpose of this work was to investigate the taxonomic status, distribution and relative abundance, habitat requirements, and nature and degree of potential threats to populations of endemic central Texas salamanders belonging to *Eurycea* and *Typhlomolge* genera (Family: Plethodontidae).

Part I is a study of the systematic relationships among *Eurycea* and *Typhlomolge* salamander population groups based on phenetic and phylogenetic analyses of 25 electrophoretic allozymes. This work represents the first comprehensive attempt to elucidate the evolutionary and systematic relationships among these salamanders. The study recommends that all population groups be placed under *Eurycea* and the genus *Typhlomolge* no longer be recognized. Population groups identified in this study should be targeted for conservation management, with conservation of spring and cave habitats and maintenance of water quality being the key factors in the survival for these salamanders.

Part II addresses systematic relationships of *Eurycea* population groups based on mitochondrial DNA (mtDNA) sequence information. The results of mtDNA analysis show a high degree of congruence, for the major groups within the central Texas *Eurycea*, with previous studies using allozymes, nuclear DNA, genome size, and in some cases, morphological data. The results reinforce the conclusion that populations north of the Colorado River are highly distinct from those to the south. On the basis of mtDNA, genome sizes, and nuclear DNA studies, this report recommends that the northern group be treated as a completely different species group from other Texas *Eurycea* for taxonomic and conservation purposes.

Part III is a report on the threats facing *Eurycea* in central Texas, north of the Colorado River. The study addresses three species within the northern group: The Jollyville Plateau Salamander, the Georgetown Salamander, and the Salado Springs Salamander. These species occur in Travis, Williamson, and Bell Counties, respectively. Major categories of impacts identified are: land disposal of waste materials, water wells, sewage and waste water disposal systems and municipal collection lines, leaks and spills, oil, gas, and mining activities, agricultural practices, ground-water withdrawals, and other factors. Management recommendations were developed elsewhere (Ref. 102; Part III) based on the information contained in these reports (Parts I, II and III). Management recommendations center on the maintenance of watershed integrity and delineation and protection of aquifer recharge zones.

# Relationships, status, and distribution of central Texas hemidactyliine plethodontid salamanders (*Eurycea* and *Typhlomolge*)

Section 6 Report Part I

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## INTRODUCTION

In this report, we will discuss the status and distribution of central Texas salamanders of the genera *Eurycea* and *Typhlomolge* (Plethodontidae: Hemidactyliini), based on studies of molecular and morphological differentiation in the group. In particular, we will focus on the major population groups and species that we have identified, primarily through the use of starch-gel electrophoresis of proteins, restriction site analysis of nuclear ribosomal DNA, flow cytometric analysis of nuclear DNA content, and morphometric analyses of external morphology. We also are sequencing portions of the mitochondrial genome; this work still is in progress and has been somewhat problematic from a technical standpoint. However, with experience and the application of new sequencing methods, our ability to gather mitochondrial sequence data for these salamanders has improved greatly, especially for an approximately 400 base pair portion of the mitochondrial cytochrome b gene. We anticipate that the cytochrome b data set soon will be complete for representatives of all the major population groups; we also are investigating the use of the mitochondrial cytochrome oxidase gene, which can readily be amplified (using the polymerase chain reaction) for these salamanders.

Because the mitochondrial data are incomplete, this report should be regarded as "part I" of a two-part series. In the second report, we will summarize the mitochondrial information and discuss this evidence in the context of all the data that we have gathered. The information that is available so far has led to major insights into differentiation and distribution of central Texas hemidactyliines; this new understanding of the degree of fragmentation in the group forms the basis for conservation strategies that will protect the diversity in the group and the fragile spring and cave habitats that these salamanders occupy.

In this report, we do not address threats to the various species and population groups of Texas hemidactyliines; some are obvious (e.g. declines in spring flow, pollution of aquifer waters) and well recognized. We feel that it is inappropriate to assess threats until all of the relevant information on relationships in the group is available, so that we can identify the targets for which conservation measures may be necessary. In the second part of this report, we will offer an assessment of threats to different taxa and groups of populations throughout central Texas.

## GENERAL INFORMATION ON CENTRAL TEXAS HEMIDACTYLIINES

Salamanders of the genera *Eurycea* and *Typhlomolge* (the latter a genus that we will no longer recognize) inhabit the southern and eastern portions of the Edwards Plateau region of central Texas, from Bell County in the northeast to Val Verde county in the southwest [see Chippindale et al. 1991 and Sweet (1978a, 1982) for maps of the distribution of the group]. This is an area of uplifted limestones that has a high concentration of springs and water-filled caves. With the exception of a few populations (most in the relatively mesic canyons of Bandera County), no members of the group are known to undergo natural transformation; instead they retain external

gills and other larval morphological features throughout their lives. Thus, members of the group are totally dependent on reliable flow of clean water from springs and/or maintenance of water in cave habitats.

When we initiated this study, the taxonomy of Texas hemidactyliines was somewhat confused, and only a few species were formally recognized:

1) *Eurycea neotenes*: formerly thought to be the most wide-ranging member of the group; Sweet (1978a, 1982) assigned the vast majority of spring and cave populations from Bell Co. to Val Verde Co. to this species.

2) *E. tridentifera*: a morphologically specialized cave form known from several caves in the Cibolo sinkhole plain region of Comal, Bexar, and perhaps Kendall Counties.

3) *E. nana*: a morphologically distinct form endemic to San Marcos Springs, Hays Co.; Sweet (1978a) suggested that this species might also occur at Comal Springs in New Braunfels, but this clearly is not the case (see below for details).

4) *Typhlomolge rathbuni*: an extreme troglobite apparently endemic to the San Marcos Pool of the Edwards Aquifer (see below for a discussion of the taxonomic status of this genus).

5) *T. robusta*: represented by a single specimen collected in 1951 from an opening in the bed of the Blanco River.

Sweet (1978, 1984) argued that two other species, *E. latitans* from the Cascade Caverns system in Kendall Co. and *E. troglodytes* from Valdina Farms Sinkhole in Medina Co., represent hybrid swarms derived from *E. tridentifera* and surface *E. neotenes*; thus he regarded these names as invalid. Sweet (1978) also argued for suppression of the name *E. pterophila*, which had been applied to members of a population from Fern Bank Springs in the Blanco River drainage of Hays Co. Recently, we described a new species (*E. sosorum*) endemic to Barton Springs in Travis Co. (Chippindale et al. 1993); we plan to describe additional species (primarily from the region to the north of the Colorado River) once the mitochondrial sequence data are complete.

In this report, we will summarize evidence that indicates that many more distinct evolutionary lineages of central Texas hemidactyliines exist than had previously been recognized. With respect to the above taxonomic arrangement, we will: (1) recommend restriction of the name *E. neotenes* to members of the populations at and in the vicinity of the type locality (near Helotes in Bexar Co.); (2) recognize the names *E. latitans* and *E. pterophila* as valid, since they refer to what appear to represent distinct evolutionary lineages; and (3) recommend that the name *Typhlomolge* should not be recognized, since members of this genus clearly are nested within the genus *Eurycea*. In addition, we will outline the compositions of the 23 groups to which we have assigned these salamanders for analytical purposes, our rationale for doing so, and the extent to which these groups are likely to represent real evolutionary entities.

## SPECIES BOUNDARIES, SPECIES CONCEPTS, AND DESIGNATION OF POPULATION GROUPS

Identification of species, and the definition of the term "species" itself, continue to be highly contentious issues, and a detailed discussion of these issues is not within the scope of this report. The species concept that we follow is that advocated by Frost and Hillis (1990), who recognized species as distinct evolutionary lineages with unique origins and histories, that are evolving separately from other such lineages. Using this definition of species, the degree of differentiation of a given lineage is not in itself what determines whether that lineage is a species; rather, the key question is whether that lineage maintains a distinct evolutionary identity from others. From a practical standpoint, measurable differentation (morphological, molecular, etc.) is very important for identification of species, because if differentiation is present it can provide evidence that one is dealing with a distinct lineage. However, other factors (e.g. geographic isolation) should also be taken into account, and therefore the decision to recognize a lineage as a species rests on consideration of all relevant information. This contrasts with some other species concepts under which discernable features of the organisms are regarded as the factors that determine whether the organisms are species or not. Examples include the "phylogenetic" species concepts (see Frost and Hillis 1990 for a review), under which species are viewed as groups of interbreeding individuals that are characterized by (and defined by) unique, evolutionarily derived features. Highton (1989) took an extreme position in his taxonomy of salamanders of the *Plethodon glutinosus* group, in which the "species" were defined by their relative genetic distances to others (i.e. a Nei's genetic distance cutoff was used, and only groups with at least this level of differentiation were considered species).

While we regard such strictly character-based approaches to recognition of species as essentially arbitrary, we have included criteria such as genetic distance and possession of unique alleles, restriction sites, etc. in our recognition of informal population groups, along with geographic and hydrogeological considerations. The groups that we will describe in the section entitled "Population groups" represent the working units that we have used in phylogenetic analyses of relationships in the Texas group. Given the available information, these are the groups that could be targeted for conservation, with conservation of spring and cave habitats and maintenance of water quality the key factors in the survival of these salamanders. We will refer to each by name, using the scientific name of a member of the group if one is available. Some of these groups may contain more than one species; we have restricted the groups to single species in cases where species status is relatively unambiguous (i.e. there is overwhelming evidence that we are dealing with a distinct evolutionary lineage). Examples include *Eurycea nana* and *E. sosorum*, each of which occupy well-defined subregions of outflow of the Edwards Aquifer, are morphologically distinct from other populations, and also can be distinguished based on molecular markers.

## SAMPLING AND PHENETIC AND PHYLOGENETIC ANALYSES

We have gathered allozyme data for the products of 25 enzyme-encoding loci for 360 individuals from 67 localities throughout the range of central Texas hemidactyliine salamanders, plus appropriate outgroup taxa for phylogenetic analysis. This sampling is very comprehensive and represents all described Texas taxa except *Eurycea troglodytes* [which Sweet (1978, 1984) regarded as an invalid taxon, and which may be extinct due to flooding of its only known subterranean habitat], and *Typhlomolge robusta* (known from a single specimen collected in 1951; presumed habitat now inaccessible). Apart from these taxa, the only other key omission is the lack of material from Fourmile Cave in Del Rio, at the extreme southwestern edge of the range of the group; we have explored this cave on several occasions but have never been able to locate salamanders, and other cavers have had similar recent experiences at this site (A.G. Grubbs, personal communication). Precise locality data for each of these sites are listed in Appendix 1; many (especially in the region north of the Colorado River) represent occurrences of these salamanders unknown prior to this study.

To represent the overall pattern of genetic similarities among salamander populations, we used UPGMA clustering of Rogers' (1972) genetic distances (results shown in Fig. 1). With few exceptions, we kept data for each individual locality separate; however, the upper limit for the Biosys-1 program that we used is 60 populations, so we combined several populations that occur in close proximity to one another and are very similar genetically, specifically: Pedernales Springs #1 and 2 (Travis Co.); Stillhouse Hollow and Barrow Hollow Springs (Travis Co.); Murphy's and Sabinal Canyon Springs (Bandera Co.); Greenwood Springs #1, 2, and 3 (Real Co.); Cherry and Cloud Hollow Springs (Kendall Co.); and the Rattlesnake Cave, Ezell's Cave, and Aquarena Springs localities for *Eurycea rathbuni*. This reduced the number of populations and taxa for analysis to 59. We also coded two loci that showed no activity in any of the individuals surveyed for specific populations or taxa (glutathione reductase in *T. rathbuni* and malate dehydrogenase 2 in Greenwood Ranch *Eurycea*) as though they were homozygous for unique alleles for these populations. Our reasoning was that, for the purposes of a similarity-based analysis, this lack of activity represents real information, although, since heterozygosity cannot be detected in cases of no activity, this could slightly bias heterozygosity estimates for these two populations. (For phylogenetic analyses, we simply coded these two loci in these two populations as "missing data", since these analyses allow this option whereas the phenetic analysis does not).

The phenetic analysis (Fig 1) reveals a strong split between the groups north and south of the Colorado River; major divisions south of the Colorado include the strong differentiation of *Eurycea rathbuni* and *E. nana*, and a further division between a southeastern and a southwestern group of populations, east and west of an imaginary line that extends from (for example) Sabinal to Fredericksburg. This is a result that we have seen before (Chippindale et al. 1992, 1993); we have narrowed the apparent distributional gap between the southeastern and southwestern groups considerably through additional collecting, but all the new "gap" populations fall phenetically within

the southeastern group. Thus it still is not clear precisely where the break in gene frequencies lies, or whether it is sudden or clinal.

A phenetic analysis of genetic distances will not necessarily reflect the evolutionary history of a given group, because (in part) taxa or populations that are highly divergent may be placed distant on the tree from others to which they are actually closely related. To investigate the evolutionary relationships of the Texas salamanders, we carried out a phylogenetic (parsimony) analysis using the program PAUP (Swofford 1990). Due to the computational intensity of the analysis, it was necessary to reduce the number of groups for analysis to a manageable number by combining various populations into the informal groups that we detail in the next section. As an outgroup (to root the tree), we used several representative hemidactyliine taxa from outside the Texas group (*Eurycea longicauda, E. bislineata, E. wilderae, E. multiplicata, E. quadridigitata* from Texas and South Carolina, *Haideotriton wallacei,* and *Typhlotriton spelaeus*). We conducted the analysis by coding for unique arrays of allele frequencies at each locus; we used Biosys-1 to calculate the Manhattan (Prevosti) distances among frequency arrays and then used these distances as the numbers of "steps" among character states (implemented through use of PAUP's stepmatrix option). This new method of analysis will be described in more detail by Chippindale et al. (in preparation).

A single tree was recovered using the heuristic search option in PAUP (Fig. 2); the pattern of relationships among groups differs in some respects from the pattern of genetic similarities revealed by the phenetic analysis. A monophyletic southern group (all populations south of the Colorado River) still appears, with *E. rathbuni* sister to all other southern forms, and distinct southeastern versus southwestern clades . However, *E. nana* falls into the southwestern group, a surprising result that is at odds with its occurrence at San Marcos Springs, in the southeastern region. This may be due to the fact that *E. nana* is highly divergent from other southern taxa, and has several unique alleles that provide no information on its relationships to other members of the Texas group. Also, it shares a 6-phosphogluconate dehydrogenase allele with members of the western group that appears to be absent in most members of the southeastern group. Another surprising result that differs from earlier analyses and from the pattern of similarities shown in the phenogram is that the northern assemblage appears as paraphyletic (i.e. the northern groups of populations do not form a cohesive group derived from a single common ancestor). Thus, despite the fact that all members of the northern group are very similar to one another allozymically and extremely divergent from the southern group (and share characteristic rDNA restriction sites and large genome sizes, information not included in this analysis), there is still considerable question as to the relationships among them. One possibility is that the group is in fact monophyletic, and the root of the tree has simply been placed incorrectly (if the root actually belongs on the branch that connects Testudo Tube to the southern group, the northern group would then appear as monophyletic). We anticipate that the mitochondrial sequence data will help to shed light on this problem; we also are experimenting with methods of analysis that will allow us to combine all the data in a single simultaneous analysis.

What is apparent from the analyses that we have carried out is that what was once considered *Eurycea neotenes* is not a monophyletic group (i.e. the populations that have been called *E. neotenes*, exclusive of other Texas *Eurycea*, do not form an evolutionary group descended from a single common ancestor), and we recommend that this name be used only for the populations at and near the type locality (near Helotes, Bexar Co.). This means that many of the other members of the group are without valid names; we are working to identify the species that are involved and name them accordingly. Given this situation, it makes sense to resurrect the names *E. latitans* and *E. pterophila*, since both of these populations are geographically distant and genetically distinct from "true" *E. neotenes*. We note that the name *E. troglodytes* may also be available for members of the southwestern group; however, the only available material for molecular work consists of approximately 20-year old tissue homogenates that are unsuitable for allozyme work and have not yet yielded usable DNA. Thus the relationships of this putative (and perhaps extinct) taxon are unclear. We will discuss our proposed name changes in detail below.

## POPULATION GROUPS

The following is a list of the population groups that we informally recognize, and a description of the composition, distribution, and characteristics of each. Where possible, we have used an existing scientific name for a member or members of the group; in other cases we have simply used the locality name of one of the group members. We stress that this arrangement may change in light of new (especially mitochondrial sequence) data; these groupings represent a "working hypothesis" only. Precise details of localities are listed in Appendix 1.

### North of the Colorado River

As discussed above, all members of the assemblage of populations to the north of the Colorado River are extremely distinct from those to the south, and similar to one another based on allozymes, rDNA restriction sites, and genome size. However, relationships among these populations still are unclear; in particular, the cave forms show some unusual allele frequency patterns and (in some cases) distinctive morphologies that cause us to treat them separately. The informal groups that we recognize among the northern populations are as follows:

**Jollyville Plateau group (Travis Co.; Williamson Co.?):**

This is a group of populations associated with the margins of the Jollyville Plateau that shows a high degree of molecular and morphological uniformity and likely represents a single species. We plan to describe this as a new species, pending completion of the mitochondrial data set. Members include the following populations: Balcones Park Spring, Barrow Hollow Springs, Bull Creek Springs, Hanks' Tract Springs, Canyon Creek Springs, Canyon Vista Spring, Horsethief Hollow Spring, Schlumberger Spring,

Stillhouse Hollow Springs, and Wheelis Tract Springs. Many new localities are likely to be discovered, especially in the Bull Creek drainage. However, all the known localities are vulnerable to damage and degradation of water quality due to increasing development in the region.

**Round Rock (Williamson Co.):**

We have collected specimens from a single spring along Brushy Creek; morphologically and based on molecular markers, these salamanders are similar to the Jollyville Plateau populations and may represent the same species. We separated them primarily on geographic grounds, since they apparently are physically isolated from the Jollyville group. Sweet (1978, 1982) also reported a population of *Eurycea* at nearby Krienke Spring, but this population apparently has been destroyed by development.

**Kretschmarr Salamander Cave (Travis Co.):**

Located on the Jollyville Plateau, salamanders in this tiny stream cave appear morphologically similar to surface populations in the area and may represent the same species. They are distinguished from spring populations in the Jollyville Plateau region allozymically by a high-frequency glucose-6-phosphate isomerase allele that otherwise is rare in the area.

**Testudo Tube (Williamson Co.):**

Salamanders in this cave appear morphologically similar to animals from surface populations, unlike individuals from the nearby Buttercup Creek Cave system which show pronounced troglobitic morphologies. Surface populations of *Eurycea* occur in springs on the nearby Audubon property (Chippindale et al. 1992); however, we have been unable to collect animals from the Audubon localities for comparison to those from Testudo Tube. This may represent an occurrence of the Jollyville Plateau group.

**Buttercup Creek Cave System (Williamson Co.):**

We have chosen to group together individuals from Buttercup Creek Cave, Twasa Cave, Ilex Cave, and Treehouse Cave, because this series of caves is well defined geographically, apparently all are hydrologically connected (Russell 1993), and adult salamanders from throughout the system show a strong troglobitic morphology (e.g. reduced eyes and pigmentation). There is substantial allozyme variation in this group, and not all members cluster together phenetically. However, sample sizes for each of these caves are very small and thus there is a high probability of sampling (and thus clustering) error for this group, particularly given the low levels of genetic differentiation that characterize most members of the northern assemblage. Our working hypothesis is that this group represents a distinct species.

**Bat Well (Williamson Co.):**

Little is known about salamanders at this locality, and we have only been able to obtain a single specimen whose affinities are unclear. This specimen possessed a Peptidase D allele that also characterizes the Georgetown and Salado population groups, but lacked the aconitate hydratase 1 and creatine kinase 1 alleles that occur at medium frequency in the Georgetown group, as well as the alpha glycerol-3-phosphate dehydrogenase allele that further characterizes members of the Georgetown group. On 8 February 1994, one of us (AHP) collected salamanders from a nearby spring in the San Gabriel River watershed (Cowan Creek Spring, 30°43'13"N, 97°44'10" W); comparison of these specimens to the Bat Well animal may shed further light on its status.

**Lake Georgetown area (Williamson Co.):**

Salamanders from springs in the vicinity of Lake Georgetown display a unique combination of alleles that distinguish them from other members of the northern assemblage of populations, specifically unique alleles at medium frequency at the aconitate hydratase 1 and creatine kinase 1 loci and an apparently fixed unique allele at the alpha glycerol-3-phosphate dehydrogenase locus. Preliminary investigations suggest that these salamanders can also be distinguished from other members of the northern assemblage based on characteristics of the lateral iridophore rows. We informally regard members of this group (represented by Avant's, Buford Hollow, Crockett Garden, and Cedar Breaks Hiking Trail Spring), as a distinct species, pending completion of the molecular work. The status and relationships of the population in the riverside springs in the park within the city of Georgetown are uncertain; these springs have been heavily modified and appear unlikely to support a healthy population of salamanders. We collected one tiny juvenile at the middle of the three park springs in 1992, but were unable to resolve the key loci due to the tiny size of the specimen.

**Salado Springs (Bell Co.):**

Salamanders from these springs are very distinctive morphologically, with elongate bodies, large rectangular heads, uniform brown to gray-brown coloration, and very reduced eyes. Discriminant morphometric analyses (Chippindale et al 1991) readily separate individuals from this population from those from other surface populations. The Salado salamanders also share a peptidase D allele with animals from the Georgetown group, an allele that otherwise appears to be very rare in the northern region. Based on the available information (primarily morphology and distribution), we regard the Salado group as a distinct species and plan to describe it as such.

**<u>South of the Colorado River</u>**

**<u>Southeastern groups</u>**

***Eurycea sosorum* (Travis Co.; Barton Springs ):**

Salamanders from Barton Springs clearly represent a distinct species, and this species recently was formally described; details were provided by Chippindale et al. (1993).

***Eurycea pterophila* group (Blanco, Hays, and Kendall Counties; Blanco River drainage):**

This group of populations shows a relatively high degree of cohesiveness based on allozyme data and geographic distribution, and we will formally resurrect the name *E. pterophila* (formerly applied to the Fern Bank population), which Sweet (1978b) regarded as invalid. Members of this group include populations at Fern Bank Springs, Peavey's Springs, Grapevine Cave, and T Cave.

**Comal Springs (Comal Co.):**

Sweet (1978) suggested that the population at Comal Springs might represent *E. nana*, but allozyme evidence (Chippindale et al. 1993) and morphometric analyses (Chippindale et al., unpublished) indicate that the populations at San Marcos and Comal Springs are readily distinguishable from one another and clearly are not conspecific. The Comal Springs population appears to be geographically isolated from others; morphologically these salamanders are similar to those from many other spring populations. Allozymically, Comal springs salamanders share a medium-frequency aconitase-1 allele that otherwise has been detected only in *Eurycea rathbuni*. The Comal Springs population may well represent a distinct species that displays relatively little morphological or molecular differentiation from other southern forms, and we currently are investigating this possibility further.

**Pedernales group (Travis Co.):**

This group was discovered in the course of this study, and the full extent of its distribution is unknown; it appears to be geographically isolated from other populations of *Eurycea*. The two known localities are springs adjacent to the Pedernales River directly across from Westcave Preserve; we have searched springs and caves on the Preserve for salamanders with no success. These are small salamanders, and in these populations a distinctive "golden" color morph is common. Apparently unique alleles at the Ldh-A and Mdhp loci occur at medium frequency, and we suspect that this group represents a distinct species.

***Eurycea latitans* group (Comal, Kendall, and Hays Counties):**

This is one of the most heterogeneous groups that we informally recognize here, and includes the following populations: Pfeiffer's Water Cave, Bear Creek Springs, Cibolo Creek Tributary Spring, Kneedeep Cave Spring, Honey Creek Cave Spring, Less Ranch

Spring, Cherry Creek Spring, Cloud Hollow Springs, and Rebecca Creek Spring. This is largely a grouping of convenience, based on overall similarity in gene frequencies, and may contain multiple species. We recognize this group as the *latitans* group because this name is available; Sweet (1978, 1984) regarded the name as invalid because he believed *E. latitans* to be hybrids between *E. neotenes* and *E. tridentifera*. However, in an allozyme survey that included five individuals from Pfeiffer's Water Cave (adjacent and hydrologically connected to the type locality for *E. latitans*, Cascade Caverns), we found these salamanders markedly different in allele frequencies from *E. tridentifera* from three different localities (Honey Creek Cave, Ebert Cave, and Badweather Pit). In particular, the *latitans* lacked a diagnostic NADP-dependent malate dehydrogenase allele that appears to be fixed or near-fixed in populations of *E. tridentifera*. Thus, it seems unlikely that this population is a hybrid swarm and (since it also does not appear to represent *E. neotenes*) the only logical solution is to reinstate the name *E. latitans*.

***Eurycea tridentifera* group (Comal and Bexar Counties; Kendall Co.?):**

This group includes morphologically specialized troglobites that form a fairly homogeneous group based on morphometric analyses (Sweet 1978a, 1984). Allozyme evidence for individuals from three populations (Honey Creek Cave, Ebert Cave, and Badweather Pit) supports Sweet's conclusion that this is a genetically relatively cohesive group and is likely to represent a single species. Refer to Sweet (1977) and Chippindale et al. (1993) for additional localities at which *E. tridentifera* is thought to occur.

***Eurycea neotenes* group (Bexar Co.):**

Members of the Helotes Creek Spring, Leon Springs, and Mueller's Spring populations cluster together based on similarities in allele frequencies, and are distinguished from other populations in part by rare alleles at the gluose-6-phosphate isomerase and phosphoglucomutase loci. Since the Helotes Creek Spring site represents the type locality for *E. neotenes* and this group forms a well-defined geographic assemblage, we regard the members of this group as the only "true" *Eurycea neotenes*. Based on the evidence thus far, application of this name to other central Texas *Eurycea* is inappropriate, especially since other named species appear to cluster phylogenetically within the group formerly assigned to *E. neotenes*.

<u>Southwestern groups</u>

**Camp Mystic (=Edmunson Creek Spring; Kerr Co):**

Animals from this locality are characterized by unique, apparently fixed alleles at the malate dehydrogenase 1 and pyruvate kinase loci, and thus are distinct genetically from other populations that we have examined. Morphologically they appear superficially similar to individuals from other populations in the region, and the taxonomic status of this population is uncertain.

**176 Spring (Kerr Co.):**

We chose to separate this population from others due to a moderate degree of genetic differentiation from other populations in the area, primarily at the alpha glycerol-3-phosphate locus. The taxonomic status of this population is uncertain.

**Greenwood Valley Ranch Springs (Real Co.):**

These three springs are near the northwestern edge of known range of *Eurycea* in the Edwards Plateau region. Salamanders from this area are characterized by a distinct allele at the isocitrate dehydrogenase 1 locus and lack of activity at the malate dehydrogenase 2 locus, and thus are distinct genetically from the other populations that we have examined. The taxonomic status of this group is uncertain.

**Sabinal River Springs (Bandera Co.):**

Salamanders from the two springs placed in this grouping, Sabinal Canyon Spring and Murphy's Spring, are characterized primarily by an otherwise rare allele at the NADP-dependent malate dehydrogenase locus. Salamanders from one of these localities (Murphy's Spring) are known to undergo natural metamorphosis (Sweet 1977). The taxonomic status of this group is uncertain.

**Tucker Hollow Cave (Real Co.):**

Salamanders in this tiny cave exhibit a distinctive morphology similar in some respects to that of individuals from the Carson Cave population (see Sweet 1978, 1984 for details of morphometric analyses). Individuals from this locality also possess a characteristic allele at the isocitrate dehydrogenase 1 locus. This population may represent a distinct species.

**Carson Cave group (Edwards, Gillespie, Kerr, and Uvalde Counties):**

Like the "*latitans* group", this is a heterogeneous assemblage of populations that we have grouped together based primarily on similarity in allele frequencies. More than one species may be involved. Included are the following localities: Carson Cave, West Nueces Spring, Sutherland Hollow Spring, Dutch Creek Spring, Robinson Creek Spring, Wetback Spring, Trough Spring, and Fessenden Springs. Individuals from the Carson Cave population are very large and exhibit a troglobitic morphology distinct from other members of this group; this population has sometimes been regarded as a distinct species based on its morphology (J. Reddell, personal communication). Individuals from the Sutherland Hollow and possibly Carson Cave localities are known to undergo natural transformation (Sweet 1977, 1978a). More investigation of this group is necessary to determine the status of its component populations.

Other southern groups:

*Eurycea nana* (Hays Co., San Marcos Springs):

We already have discussed the status of this species in this report (see description of the Comal Springs group) and in others (e.g. Chippindale et al. 1993). It clearly represents a distinct species, restricted to the outflows of San Marcos Springs.

*Eurycea* (formerly *Typhlomolge*) *rathbuni* (Hays Co., subterranean waters at San Marcos):

This is another distinct species that is readily distinguishable based on morphology and molecular evidence. It appears to restricted to the San Marcos Pool of the Edwards Aquifer. Relationships of the presumed sister taxon *E.* (formerly *T.*) *robusta* are uncertain, due to lack of availability of fresh specimens. We follow Mitchell and Reddell (1965) and Mitchell and Smith (1972) in use of the name *Eurycea rathbuni*, since the molecular evidence indicates that this species is nested phylogenetically within the Texas *Eurycea*.

## CONCLUSIONS

The arrangement of groups within the Texas *Eurycea* that we have suggested here leaves many evolutionary and taxonomic problems in the group unsolved. However, this represents the first comprehensive attempt to recover the evolutionary history and determine relationships of the group, which has proven to be highly fragmented and extremely diverse at both the morphological and molecular levels. We anticipate that the additional information that we are in the process of gathering will further resolve these problems, but given the nature of this group, it is likely that many questions will remain. In part II of this report, we will provide further information on the status of the working groups that we have identified, and recommend strategies for conservation of these salamanders to protect the genetic diversity that exists in this complex assemblage.

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# FIGURE LEGENDS

Figure 1: Phenetic clustering of populations of Texas *Eurycea*, based on allozyme electrophoresis of the products of 25 enzyme-encoding loci. Informal groups that we recognize are indicated in capital letters; the Buttercup Creek Cave group contains Buttercup Creek, Ilex, Treehouse, and Twasa Caves. See text for additional details of this analysis.

Figure 2: Phylogenetic (parsimony) analysis of working groups of Texas *Eurycea*, using frequency-based coding of allelic composition. Refer to text for additional details of this analysis. Note that the rooting (which renders the northern group paraphyletic) is questionable (see text for discussion).

## Figure 1, part 1 (phenetic clustering of northern populations)

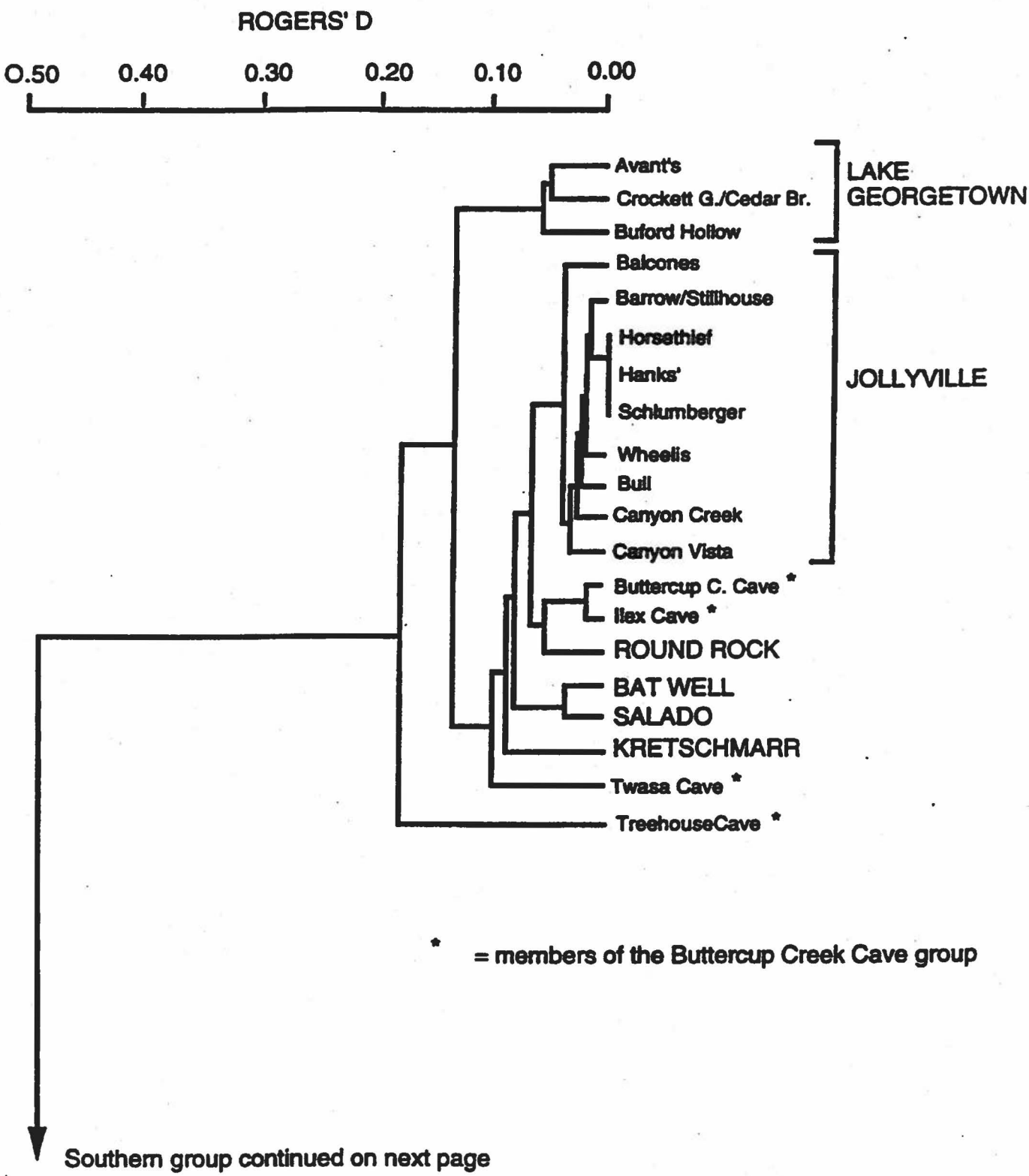


Figure 1 continued

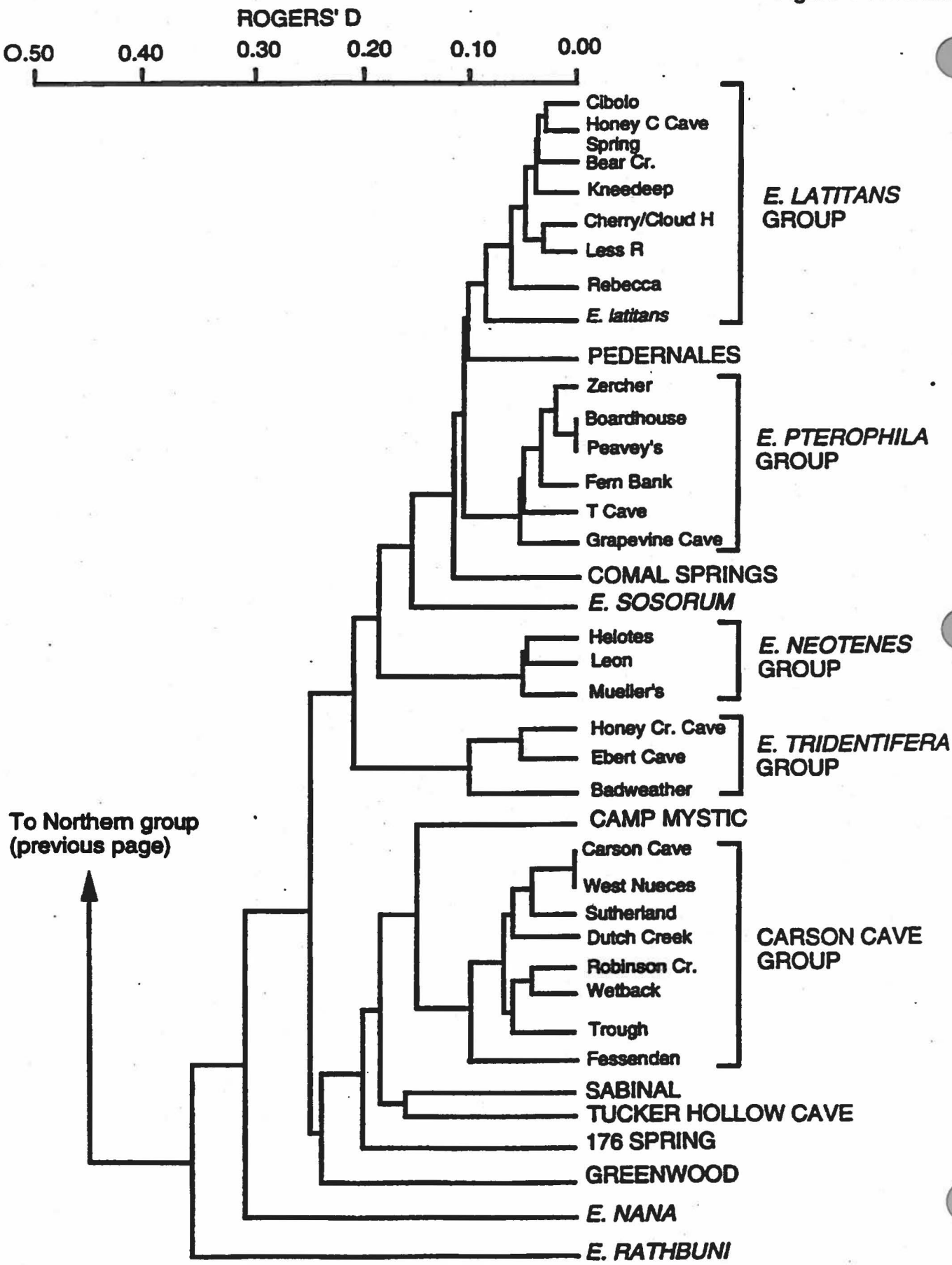
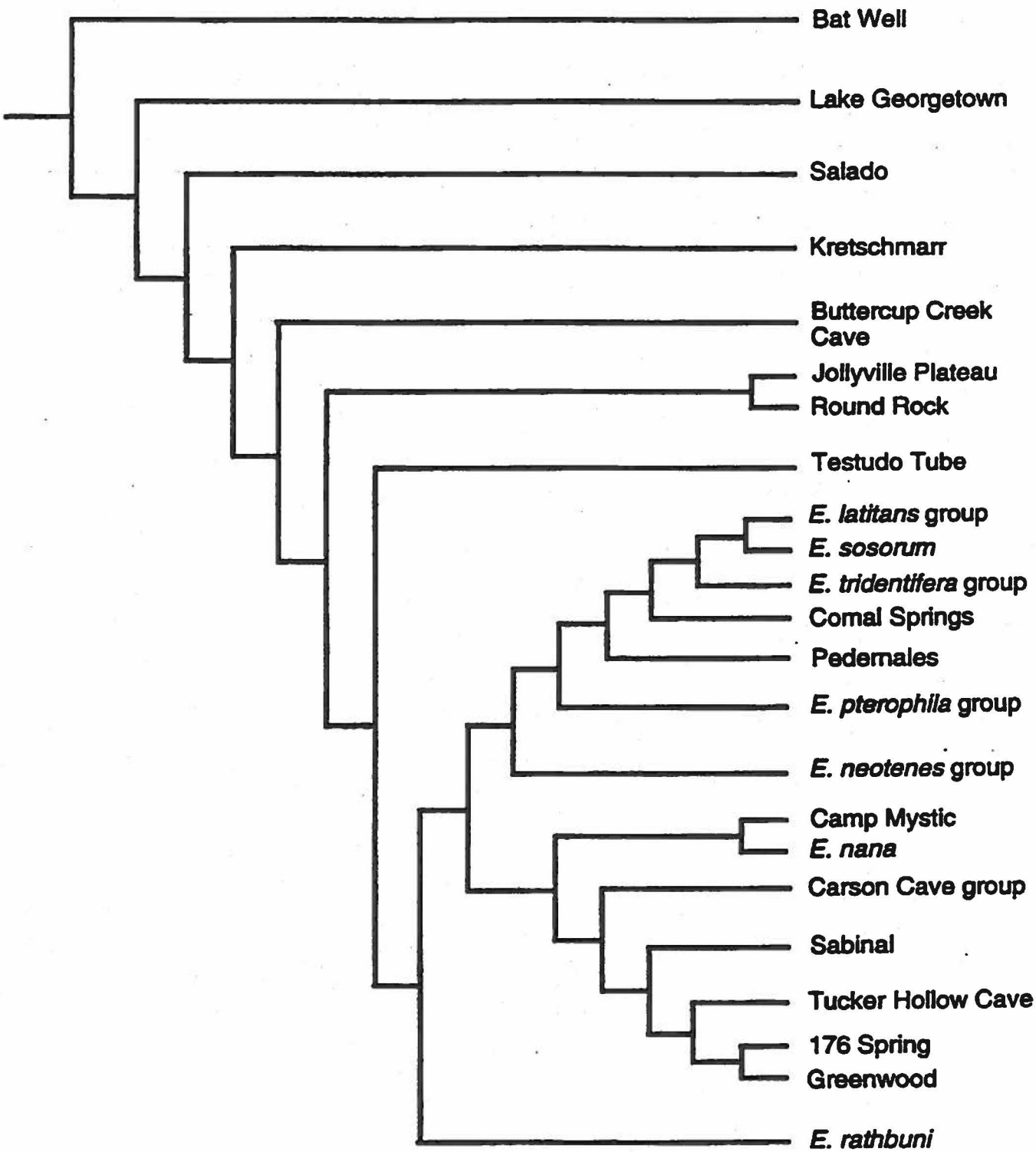


Figure 2


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

# APPENDIX I: LOCALITIES SAMPLED IN THE COURSE OF THIS STUDY

**APPENDIX 1.** Specimen localities for population groups of central Texas hemidactyliine salamanders cited in this report. Watersheds in which each locality are situated are given where not obvious. The localities from which specimens have previously received a formal taxonomic designation are indicated.

**BANDERA COUNTY**

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

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

3. Sutherland Hollow Spring, west prong Medina River, 29°44'58" N, 99°25'36" W.

**BELL COUNTY**

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

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

**BEXAR COUNTY**

6. Helotes Creek Spring, Medina River watershed, 29°38'15" N, 98°41'40" W.

7. Leon Springs, Leon Creek, Medina River watershed, 29°39'46" N, 98°38'14" W.

**BLANCO COUNTY**

8. Zercher Spring, Blanco River, 30°06'10" N, 98°27'25" W.

9. Boardhouse Springs, Blanco River watershed, 30°06'40" N, 98°18'07" W.

10. T-Cave, Blanco River watershed, 30°04'36" N, 98°19'46" W.

**COMAL COUNTY**

11. Rebecca Creek Spring, Guadalupe River watershed, 29°55'28" N, 98°22'22" W.

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

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

14. Honey Creek Cave, Guadalupe River watershed, 29°50'50" N, 98°29'30" W. (*Eurycea tridentifera*).

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

**EDWARDS COUNTY**

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

17. West Nueces River Spring, 29°43'20" N, 100°24'51" W.

**GILLESPIE COUNTY**

18. Trough Spring, Pedernales River watershed, 30°08'36" N, 99°04'40" W.

**HAYS COUNTY**

19. Grapevine Cave, Blanco River watershed, approximately 30°02'30" N, 98°12'45" W.

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

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

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

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

**KENDALL COUNTY**

24. Bear Creek Spring, Medina River watershed, 29°48'15" N, 98°52'10" W.

25. Cibolo Creek Tributary Spring, Cibolo Creek watershed, 29°49'03" N, 98°51'43" W.

26. Less Ranch Spring,

27. Peavey's Springs, headwaters of the Blanco River, approximately 30°05'30" N, 98°39'30" W.

28. Kneedeep Cave Spring, Guadalupe River State Park, 29°52'31" N, 98°29'05" W.

29. Pfeiffer's Water Cave, Guadalupe River watershed, 29°45'44" N, 98°39'59" W. (*Eurycea latitans*).

30. Mueller's Spring, Medina River watershed, approximately 29°44' N, 98°47'30" W.

**KERR COUNTY**

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

32. Fessenden Springs, Guadalupe River watershed, 30°10'00" N, 99°20'32" W.

33. 176 Spring, Guadalupe River watershed, 30°05'18" N, 99°19'14" W.

34. Robinson Creek Spring, north prong Medina River watershed, 29°54'55" N, 99°15'08" W.

35. Cloud Hollow Spring,

36. Cherry Creek Spring,

**REAL COUNTY**

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

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

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

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

**TRAVIS COUNTY**

41. Balcones Community Park Spring, Walnut Creek watershed, 30°24'45" N, 97°43'02" W.

42. Barrow Hollow Spring, Bull Creek watershed, 30°22'33" N, 97°46'02" W.

43. Stillhouse Hollow Springs, Bull Creek watershed, 30°22'28" N, 97°45'55" W.

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

45. Bull Creek Spring Pool, west fork Bull Creek, 30°24'59" N, 97°49'00" W.

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

47. Canyon Creek Spring, north fork Bull Creek, 30°25'33" N, 97°48'51" W.

48. Canyon Vista Spring, Bull Creek watershed, 30°25'51" N, 97°46'55" W.

49. Horsethief Hollow Spring, Bull Creek watershed, 30°24'31" N, 97°49'00" W.

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

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

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

53. Wheelis Springs, Long Hollow Creek, Colorado River watershed, 30°27'42" N, 97°52'28" W.

54. Barton Springs, Barton Creek, 30°15'49" N, 97°46'14" W. (*Eurycea sosorum*).

**UVALDE COUNTY**

55. Wetback Spring, Sabinal River watershed, 29°35'12" N, 99°36'14" W.

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

**WILLIAMSON COUNTY**

57. Avant's Spring, middle fork of the San Gabriel River, 30°38'44" N, 97°44'11" W.

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

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

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

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

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

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

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

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

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

67. Brushy Creek Spring, 30°31'00" N, 97°39'38" W.

# Relationships, status, and distribution of central Texas hemidactyliine plethodontid salamanders (*Eurycea* and *Typhlomolge*)

**Final Section 6 Report, Part II**

**July 1994**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## INTRODUCTION

In this report, we will discuss the status and relationships of central Texas cave- and spring-dwelling salamanders of the genus *Eurycea* (including the taxon that formerly was considered *Typhlomolge*), incorporating new DNA sequence data for a portion of the mitochondrial cytochrome b gene. In part I of this report (Chippindale et al. 1994) and in previous reports (Chippindale et al. 1991, 1992, 1993), we provided background information and taxonomic history for this group; this report includes some material that was included in Part I, with the exception of the detailed appendix in which specific localities for the populations that we examined were listed. Here we will focus to the greatest extent on the degree to which the mitochondrial DNA (mtDNA) data support or refute the groupings of *Eurycea* that we outlined previously based on allozymes, nuclear ribosomal DNA restriction sites, genome size, and/or morphology. We also will discuss the implications of the mtDNA findings with respect to relationships and degree of evolutionary divergence of members of this group, and suggest some populations or groups of populations that should be targeted for conservation efforts. Finally, we will offer recommendations on future research that could help to clarify some of the uncertainties that still exist regarding relationships and species boundaries in the Edwards Plateau *Eurycea*.

## GENERAL INFORMATION ON CENTRAL TEXAS HEMIDACTYLIINES

Salamanders of the genera *Eurycea* and *Typhlomolge* (the latter a genus that we will no longer recognize) inhabit the southern and eastern portions of the Edwards Plateau region of central Texas, from Bell County in the northeast to Val Verde county in the southwest [see Chippindale et al. 1991 and Sweet (1978a, 1982) for maps of the distribution of the group]. This is an area of uplifted limestones that has a high concentration of springs and water-filled caves. With the exception of a few populations (most in the relatively mesic canyons of Bandera County), no members of the group are known to undergo natural transformation; instead they retain external gills and other larval morphological features throughout their lives. Thus, members of the group are totally dependent on reliable flow of clean water from springs and/or maintenance of water in cave habitats.

When we initiated this study, the taxonomy of Texas hemidactyliines was somewhat confused, and only a few species were formally recognized:

1) *Eurycea neotenes*: formerly thought to be the most wide-ranging member of the group; Sweet (1978a, 1982) assigned the vast majority of spring and cave populations from Bell Co. to Val Verde Co. to this species.

2) *E. tridentifera*: a morphologically specialized cave form known from several caves in the Cibolo sinkhole plain region of Comal, Bexar, and perhaps Kendall Counties.

3) *E. nana*: a morphologically distinct form endemic to San Marcos Springs, Hays Co.; Sweet (1978a) suggested that this species might also occur at Comal Springs in New Braunfels, but this clearly is not the case (see below for details).

4) *Typhlomolge rathbuni*: an extreme troglobite apparently endemic to the San Marcos Pool of the Edwards Aquifer (see below for a discussion of the taxonomic status of this genus).

5) *T. robusta*: represented by a single specimen collected in 1951 from an opening in the bed of the Blanco River.

Sweet (1978, 1984) argued that two other species, *E. latitans* from the Cascade Caverns system in Kendall Co. and *E. troglodytes* from Valdina Farms Sinkhole in Medina Co., represent hybrid swarms derived from *E. tridentifera* and surface *E. neotenes*; thus he regarded these names as invalid. Sweet (1978b) also argued for suppression of the name *E. pterophila*, which had been applied to members of a population from Fern Bank Springs in the Blanco River drainage of Hays Co. Recently, we described a new species (*E. sosorum*) endemic to Barton Springs in Travis Co. (Chippindale et al. 1993); we also plan to describe additional species (primarily from the region to the north of the Colorado River).

In this report, we will summarize evidence that indicates that many more distinct evolutionary lineages of central Texas hemidactyliines exist than had previously been recognized. With respect to the above taxonomic arrangement, we will: (1) recommend restriction of the name *E. neotenes* to members of the populations at and in the vicinity of the type locality (near Helotes in Bexar Co.); (2) recognize the names *E. latitans* , *E. troglodytes*, and *E. pterophila* as valid, since they refer to what appear to represent distinct evolutionary lineages (*E. latitans* and *E. troglodytes* do not appear to be hybrids based on the molecular data that we have gathered); and (3) recommend that the name *Typhlomolge* should not be recognized, since members of this genus clearly are nested within the genus *Eurycea*. In addition, we will outline the compositions of the groups to which we have assigned these salamanders, our rationale for doing so, and the extent to which these groups are likely to represent real evolutionary entities. Composition of these groups, and those that we delineated earlier (in Part I of this report) will be discussed in light of the new mitochondrial sequence data.

## SPECIES BOUNDARIES, SPECIES CONCEPTS, AND DESIGNATION OF POPULATION GROUPS

Identification of species, and the definition of the term "species" itself, continue to be highly contentious issues, and a detailed discussion of these issues is beyond the scope of this report. The species concept that we follow is that advocated by Frost and Hillis (1990), who recognized species as distinct evolutionary lineages with unique origins and histories, that are evolving separately from other such lineages. Using this definition of species, the degree of differentiation of a given lineage is not in itself what determines whether that lineage is a species; rather, the key question is whether that lineage maintains a distinct evolutionary identity from others. From a practical standpoint, measurable differentation (morphological, molecular, etc.) is very important for identification of species, because if differentiation is present it can provide evidence that one is dealing with a distinct lineage. However, other factors (e.g. geographic isolation) also should be taken into account, and therefore the decision to recognize a lineage as a species rests on consideration of all relevant information. This contrasts with some other species concepts under which discernable features of the organisms are regarded as the factors that determine whether the organisms are species or not. Examples include the "phylogenetic" species concepts (see Frost and Hillis 1990 for a review), under which species are viewed as groups of interbreeding individuals that are characterized by (and defined by) unique, evolutionarily derived features. Highton (1989) took an extreme position in his taxonomy of salamanders of the *Plethodon glutinosus* group, in which the "species" were defined by their relative genetic distances to others (a Nei's genetic distance cutoff was used, and only groups with at least this level of differentiation were considered species).

While we regard such strictly character-based approaches to recognition of species as essentially arbitrary, we have included criteria such as genetic distance and possession of unique alleles, restriction sites, sequence substitutions, etc. in our recognition of informal population groups and

distinct species, along with geographic and hydrogeological considerations. Our goal is to identify populations or groups of populations that represent distinct evolutionary entities. The groups that we will describe represent the working units that we have used in phylogenetic analyses of relationships in the Texas group. Given the available information, these are the groups that could be targeted for conservation, with protection of spring and cave habitats and maintenance of water quality the key factors in the survival of these salamanders. We will refer to each by name, using the scientific name of a member of the group if one is available. Some of these groups may contain more than one species; we have restricted the groups to single species in cases where species status is relatively unambiguous (i.e. there is overwhelming evidence that we are dealing with a distinct evolutionary lineage). Examples include *Eurycea nana* and *E. sosorum*, each of which occupy well-defined subregions of outflow of the Edwards Aquifer, are morphologically distinct from other populations, and also can be distinguished based on molecular markers.

## ALLOZYMES: METHODS AND RESULTS

We have gathered allozyme data for the products of 25 enzyme-encoding loci for 360 individuals from 67 localities throughout the range of central Texas hemidactyliine salamanders, plus appropriate outgroup taxa for phylogenetic analysis. This sampling is very comprehensive and represents all described Texas taxa except *Eurycea troglodytes* (for which we do have cytochrome b sequence data, and which may be extinct due to flooding of its only known subterranean habitat), and *Typhlomolge robusta* (known from a single specimen collected in 1951; presumed habitat now inaccessible). Apart from these taxa, the only other key omission is the lack of material from Fourmile Cave and San Felipe Springs in Del Rio, at the extreme southwestern edge of the range of the group; we have investigated the cave and springs on several occasions but have never been able to locate salamanders. At Fourmile Cave, other cavers have had similar recent experiences (A.G. Grubbs, personal communication). Precise locality data for each of these sites are listed in Appendix 1 of Part I of this report; many (especially in the region north of the Colorado River) represent occurrences of these salamanders unknown prior to this study.

To depict the overall pattern of genetic similarities among salamander populations, we used UPGMA clustering of Rogers' (1972) genetic distances (results shown in Fig. 1). With few exceptions, we kept data for each individual locality separate; however, the upper limit for the Biosys-1 program that we used is 60 populations, so we combined several populations that occur in close proximity to one another and are very similar genetically, specifically: Pedernales Springs #1 and 2 (Travis Co.); Stillhouse Hollow and Barrow Hollow Springs (Travis Co.); Murphy's and Sabinal Canyon Springs (Bandera Co.); Greenwood Springs #1, 2, and 3 (Real Co.); Cherry and Cloud Hollow Springs (Kendall Co.); and the Rattlesnake Cave, Ezell's Cave, and Aquarena Springs localities for *Eurycea rathbuni*. This reduced the number of populations and taxa for analysis to 59. We also coded two loci that showed no activity in any of the individuals surveyed for specific populations or taxa (glutathione reductase in *T. rathbuni* and malate dehydrogenase 2 in Greenwood Ranch *Eurycea*) as though they were homozygous for unique alleles for these populations. Our reasoning was that, for the purposes of a similarity-based analysis, this lack of activity represents real information, although, since heterozygosity cannot be detected in cases of no activity, this could slightly bias heterozygosity estimates for these two populations. (For phylogenetic analyses, we simply coded these two loci in these two populations as "missing data", since these analyses allow this option whereas the phenetic analysis does not).

The phenetic analysis (Fig 1) reveals a major split between the groups north and south of the Colorado River; major divisions south of the Colorado include the strong differentiation of *Eurycea rathbuni* and *E. nana*, and a further division between a southeastern and a southwestern

group of populations, east and west of an imaginary line that extends from (for example) Sabinal to Fredericksburg. This is a result that we have seen before (Chippindale et al. 1992, 1993); we have narrowed the apparent distributional gap between the southeastern and southwestern groups considerably through additional collecting, but all the new "gap" populations fall phenetically within the southeastern group. Thus it still is not clear precisely where the break in gene frequencies lies, or whether it is sudden or clinal.

A phenetic analysis of genetic distances will not necessarily reflect the evolutionary history of a given group, because (in part) taxa or populations that are highly divergent may be placed distant on the tree from others to which they are actually closely related. To investigate the evolutionary relationships of the Texas salamanders, we carried out a phylogenetic (parsimony) analysis using the program PAUP (Swofford 1990). Due to the computational intensity of the analysis, it was necessary to reduce the number of groups for analysis to a manageable number by combining various populations into the informal groups that we describe in the section entitled Major Groups. As an outgroup (to root the tree), we used several representative hemidactyliine taxa from outside the Texas group (*Eurycea longicauda*, *E. bislineata*, *E. wilderae*, *E. multiplicata*, *E. quadridigitata* from Texas and South Carolina, *Haideotriton wallacei*, and *Typhlotriton spelaeus*). We conducted the analysis by coding for unique arrays of allele frequencies at each locus; we used Biosys-1 to calculate the Manhattan (Prevosti) distances among frequency arrays and then used these distances as the numbers of "steps" among character states (implemented through use of PAUP's stepmatrix option). This new method of analysis will be described in more detail by Chippindale et al. (in preparation).

A single tree was recovered using the heuristic search option in PAUP (Fig. 2); the pattern of relationships among groups differs in some respects from the pattern of genetic similarities revealed by the phenetic analysis. A monophyletic southern group (all populations south of the Colorado River) still appears, with *E. rathbuni* sister to all other southern forms, and distinct southeastern versus southwestern clades . However, *E. nana* falls into the southwestern group, a surprising result that is at odds with its occurrence at San Marcos Springs, in the southeastern region. This may be due to the fact that *E. nana* is highly divergent from other southern taxa, and has several unique alleles that provide no information on its relationships to other members of the Texas group. Also, it shares a 6-phosphogluconate dehydrogenase allele with members of the western group that appears to be absent in most members of the southeastern group. Another surprising result that differs from earlier analyses and from the pattern of similarities shown in the phenogram is that the northern assemblage appears as paraphyletic (i.e. the northern groups of populations do not form a cohesive group derived from a single common ancestor). Thus, despite the fact that all members of the northern group are very similar to one another allozymically and extremely divergent from the southern group (and share characteristic rDNA restriction sites and large genome sizes, information not included in this analysis), there is still considerable question as to the relationships among them based on the allozyme data alone. One possibility is that the group is in fact monophyletic, and the root of the tree has simply been placed incorrectly (if the root actually belongs on the branch that connects Testudo Tube to the southern group, the northern group would then appear as monophyletic). The mitochondrial sequence data shed considerable light on this problem (see below), strongly supporting the monophyly of the northern group.

What is apparent from the analyses that we have carried out using the allozyme data is that what was once considered *Eurycea neotenes* is not a monophyletic group (i.e. the populations that have been called *E. neotenes*, exclusive of other Texas *Eurycea*, do not form an evolutionary group descended from a single common ancestor), and we recommend that this name be used only for the populations at and near the type locality (near Helotes, Bexar Co.).

The conclusion that multiple species exist within what has been called *E. neotenes* also is strongly supported by the cytb sequence data (see below). This means that many of the

members of the group are without valid names; we are working to identify the species that are involved and name them accordingly. Given this situation, it makes sense to resurrect the names *E. latitans* , *E. trogodytes*, and and *E. pterophila*, since these populations are geographically distant and genetically distinct from "true" *E. neotenes*.

## MITOCHONDRIAL DNA

Sequencing of mtDNA has proven to be a powerful tool in systematics, with applications that range from study of within-species, interpopulation relationships to resolution of relationships at much higher taxonomic levels (e.g. see Kocher et al. 1989, Simon 1991). In vertebrates, the mitochondrial genome is inherited only maternally and undergoes no recombination; thus it serves as a marker for reconstruction of relationships among female lineages within or among species (Brown 1985). These relationships normally will reflect those of the populations or taxa, unless there are differential patterns of gene flow between the sexes (e.g. if males are very mobile and females are not, mtDNA may show greater geographic substructuring than many nuclear markers because the males can spread only the nuclear genome; see Moritz et al. 1992 for an example from salamanders). For the Edwards Plateau *Eurycea*, this is very unlikely to be a factor, since most populations are limited to very specific "islands" of aquatic habitat (springs and caves). Genes in the mitochondrial genome span a wide range of evolutionary rates; cytochrome b is generally regarded as evolving at an intermediate rate among vertebrates, although this varies among taxa. We chose to sequence a portion of the mitochondrial cytochrome b gene because other studies (e.g. Moritz et al. 1992) and our initial investigations suggested that this gene displays levels of divergence appropriate for investigation of relationships of the central Texas *Eurycea*. These expectations were borne out to a large extent, as we will discuss in subsequent portions of this report.

## MITOCHONDRIAL DNA: METHODS

### DNA amplification

To amplify double-stranded mtDNA from dilute samples of extracted, whole genomic salamander DNA, we used standard PCR (polymerase chain reaction) methods (see Ehrlich 1989 and Innis et al. 1990 for overviews of PCR theory and methods). Specific details of PCR conditions are available from PC and will be published separately. We experimented with various combinations of the primers described by Moritz et al. (1992) to amplify portions of the mitochondrial cytochrome b (cytb) gene, and finally settled on the combination of the primers MVZ 15 and CB2H (a truncated version of Moritz et al.'s b2 primer) as the most reliable in producing large quantities of high-quality product that was amenable to asymmetric PCR (see below). The primer sequences are:

MVZ 15 (light strand): GAACTAATGGCCCACAC(AT)(AT)TACG

CB2H (heavy strand): CCCCTCAGAATGATATTTGTCCTCA

These primers amplify an approximately 386 base-pair (bp) region of the 3' end of the gene; of this, we were able to read approximately 330-350 bp per taxon or population (sequence became uninterpretable near the primers).

Initially we used asymmetric PCR (e.g. McCabe 1990) to generate single-stranded (SS) DNA from the double-stranded PCR product, and then sequenced the SS DNA using standard Sanger sequencing methods with radioactive sulfur isotopes (e.g. Hillis et al. 1990). However, this method proved inconsistent, with the limiting step usually the asymmetric PCR, and it often took many repeated attempts to obtain readable sequence for a given taxon. Given these problems, we switched to use of cycle sequencing methods with $^{32}P$ (e.g. Murray 1989, Craxton 1991) and sequenced both strands with substantial overlap in the middle of the region of interest. This approach yielded much more consistent results after considerable experimentation with optimization of conditions. Specific details of the cycle sequencing methods used for the Texas *Eurycea* are available from PC and will be published separately.

To ensure that we were dealing with the mitochondrial cytb gene and not, for example, a nuclear cytb pseudogene, we sequenced the region of interest for a sample of purified mtDNA from a member of the Sutherland Hollow population. The sequence was identical to that for a member of the same population for which we sequenced the cytb region in the conventional way. Thus, we feel confident that we did indeed obtain sequence for the mitochondrial cytochrome b gene.

Chelex amplification of small quantities of sample

Late in the study, we experimented with use of Chelex, a chelator of divalent cations manufactured by BioRad, to amplify DNA from old (mid-1970's) allozyme homogenates that we suspected might contain usable DNA (the samples were provided by David Wake of the Museum of Vertebrate Zoology, University of California at Berkeley). We took this approach because one of the taxa involved, *E. troglodytes*, can no longer be located and may be extinct due to flooding of its only known habitat, Valdina Farms Sinkhole in Medina County (George Veni, personal communication, and Veni and Associates 1987). The method worked extremely well, not only for samples of allozyme homogenates but also for tiny quantities of tissues from other sources. This opens up the possibility for nondestructive sampling of salamanders (and other organisms); presumably even a clipped toe would be enough to yield amplifiable DNA, so one could check the identity of a live specimen based on mtDNA without harming the animal. We are pursuing this approach for future studies of the Edwards Plateau *Eurycea*. The specific technique that we used was based on an as yet unpublished protocol from the laboratory of Craig Moritz; this information is available from PC.

Outgroup composition, missing data, and populations included in the study

While our unpublished analyses indicate that the Edwards Plateau *Eurycea* constitute a monophyletic group (i.e. they are descended from a single common ancestor not shared by other *Eurycea*), the relationship of this group to other *Eurycea* and related genera is uncertain. Therefore, we included sequence for multiple taxa as outgroups (the outgroup is used as the basis for rooting the tree for the ingroup, in this case the central Texas *Eurycea*). These outgroup taxa were:

*E. bislineata*
*E. cirrigera*
*E. l. longicauda*
*E. quadridigitata* (South Carolina)
*E. quadridigitata* (Texas)
*E. m. multiplicata*
*Haideotriton wallacei*
*Typhlotriton spelaeus*

In some cases, sequence alignment for the outgroup taxa was problematic; in general we dealt with this in an interim way by treating uncertainties as "gaps", and then did not include the gaps in the analyses. Thus it is unlikely that these uncertainties for the outgroup affected the rooting of the ingroup tree substantially (although we do plan to repeat the analyses once alignments for the outgroup are clarified).

Sequences for members of the Texas group were readily and unambiguously alignable, and most have been double-checked by repeat sequencing. We caution, however, that a few uncertainties remain to be checked. Most are in the form of apparent autapomorphies (substitutions unique to a single population) and thus are unlikely to have an effect on our inferences of relationships. We are missing substantial amounts of sequence for two of the populations included in the study: the Pedernales sample has consistently failed to yield interpretable sequence when sequenced with MVZ 15, and thus we only have about 250 bp of sequence based on use of CB2H as the sequencing primer; and we are missing about half the sequence for the 176 Spring population due to a simple oversight that is easily rectified. Placements of these populations should thus be regarded cautiously (although each does fall within the expected geographic grouping based on the available data; see below for details).

We included the following populations in the study, and based our choice in part on the groupings that we identified earlier based on allozymes, nuclear ribosomal DNA restriction sites, and genome size variation (see Part I of this report). We included multiple representatives of some groups, particularly those that we suspected to be heterogeneous (especially the "*latitans*" and "Carson Cave" groups identified in part I of this report). Where appropriate, names of the group to which we tentatively assigned the populations in Part I of this report follow population names in parentheses.

Populations included:

*E.* ("*Typhlomolge*") *rathbuni*
*E. sosorum*
*E. neotenes* Helotes Creek Spring
*E. tridentifera* Badweather Pit
*E. tridentifera* Ebert Cave
*E. tridentifera* Honey Creek Cave
*E. nana*
*E. latitans* Pfeiffer's Water Cave (*latitans* group)
Rebecca Creek Springs (*latitans* group)
Cibolo Creek Tributary Springs (*latitans* group)
Honey Creek Cave Spring (*latitans* group)
Cloud Hollow Springs (*latitans* group)
Comal Springs
Boardhouse Springs (*E. pterophila* group)*
Pedernales Springs
Greenwood Springs
Sabinal Canyon Springs
Tucker Hollow Cave
176 Spring
Carson Cave (Carson Cave group)
Trough Spring (Carson Cave group)
Sutherland Hollow Spring (Carson Cave group)

Continued...

Populations included (continued)

*E. troglodytes* (not previously assigned to any group because data had been unavailable; may be extinct)
Camp Mystic Spring
Salado Springs
Cedar Breaks Hiking Trail Spring (Lake Georgetown group)
Bat Well
Stillhouse Hollow Springs (Jollyville Plateau group)
Horsethief Hollow Spring (Jollyville Plateau group)
Round Rock (=Brushy Creek) Spring (Jollyville Plateau group?)
Kretschmarr Salamander Cave (Jollyville Plateau group?)
Testudo Tube (Jollyville Plateau group? Buttercup Creek Cave group?)
Ilex Cave (Buttercup Creek Cave group)

*Note that we inadvertently omitted this and the Zercher Spring populations from the list of *E. pterophila* group localities in Part I of this report.

Refer to Appendix 1 in Part I of this report for precise details of localities for the above populations; this information also is available in a TPWD database and from AHP.

Phylogenetic analyses of mtDNA data

We used the heuristic search option in PAUP (Swofford, 1990) for parsimony analysis of the cytb sequence data. To reduce the number of populations and taxa to a manageable number for analysis (computational intensity increases exponentially with increasing number of taxa) we eliminated populations with sequences identical to those of others, or sequences that included only autapomorphies (uniquely derived changes that provide no phylogenetic information). We then added these populations and taxa to the final consensus tree from the analysis. To obtain estimates of branch lengths (which reflect the degree of divergence of particular taxa or groups), we conducted a heuristic search that included all populations, stopped the search partway through (it could easily have run for weeks) and arbitrarily chose one of the many equally most parsimonious (shortest) trees identified up to that point in the search (Figure 2). While this approach does not allow identification of the shortest tree, it should provide a good approximation of branch lengths, and makes patterns of differentiation easier to visualize than (for example) presentation of a table of distances or measures of sequence divergence.

For the analyses shown here, we weighted transitions and transversions equally. In additional analyses (not shown) in which we accorded transversions twice the weight of transitions (due to their apparent rarity) results were similar, except that overall resolution was reduced, *E. rathbuni*, *E. nana*, and the Pedernales group sometimes formed a distinct clade, and the Carson Cave population sometimes (in 31% of equally most parsimonious trees) formed part of a basal trichotomy between an otherwise monophyletic southwestern group and a monophyletic southeastern group.

## MITOCHONDRIAL DNA: GENERAL RESULTS

Of the approximately 330-350 bp of cytb sequence obtained for nearly all populations, approximately 70 sites were variable within the central Texas group. The majority of changes

appear to be transitions (purine-purine or pyrimidine-pyrimidine changes; i.e. A-G/G-A or C-T/T-C); however, we have not yet determined the proportions of substitution types rigorously. The region sequenced has proven to be extremely useful for delineation of major groups within the central Texas *Eurycea*, and for the most part there is a high degree of congruence between the results of analyses of the mitochondrial sequence data and relationships and species boundaries suggested by allozyme, nuclear ribosomal DNA restriction site, genome size, and in some cases morphological data. Where the mtDNA data are not so useful is on a fine scale in some areas; in some cases [particularly within the "southeastern" region bounded by the Colorado River to the northeast and an imaginary line between (for example) Fredericksburg and Sabinal to the west] there is little or no cytb differentiation, even among species that are distinguishable based on allozymes and morphology (e.g. *E. sosorum*, *E. tridentifera*, and other populations in the region). For these regions, it will be necessary to seek more highly variable mitochondrial and/or nuclear markers (see Conclusions and Recommendations). Although the mtDNA data cannot address all of the issues raised by previous studies, they are of great value in confirming many of the conclusions that we reached earlier, in delineating major areas and groups of populations that can be targeted for conservation efforts, and in some cases have suggested patterns of relationships not previously detected. In the following section we will discuss major population groups and species boundaries in the Edwards Plateau *Eurycea* in light if the new cytb data.

## MAJOR GROUPS

### Northern group

The cytb sequence data strongly reinforce our conclusions based on other molecular data that the populations north of the Colorado River are highly distinct from those to the south, and Figure 3 reveals the same basal split between northern and southern groups seen in phenetic analyses of the allozyme data (Part I of this report). This split also corresponds to a major break in genome sizes, and to the occurrence of novel nuclear ribosomal DNA restriction sites in the northern group. Members of the northern group can be distinguished from other central Texas *Eurycea* by differences at at least seventeen cytb sequence positions (with a few exceptions in which Salado, Testudo Tube, Ilex Cave, and Bat Well, or the Lake Georgetown populations differ at these sites); 10 of these appear to be synapomorphies for the group (i.e. are uniquely derived, not seen in other Texas populations or in outgroup members). Thus we can say with an extremely high degree of confidence that the northern group should be treated as a completely different species group from other Texas *Eurycea* for both taxonomic and conservation purposes.

However, relationships within the northern group remain problematic in several respects. None of the molecular data are sufficient to clarify the position of the cave salamanders in the region; analysis of the cytb data suggests (with relatively weak support) a grouping that includes the populations from Ilex Cave (Buttercup Creek system), Testudo Tube (near Ilex but may be hydrologically distinct; W. Russell pers. comm.), Kretschmarr Salamander Cave (Jollyville Plateau) and Bat Well (northwest of Georgetown). This is intriguing, as it suggests an early separation of the cave populations in the area from many of the surface populations, but requires further study, perhaps through use of highly variable mitochondrial markers. Salamanders from the Buttercup Creek Cave system (exclusive of Testudo Tube) also appear based on superficial examination to be distinct from other members of the northern group, and thus morphological and morphometric study is desirable. Currently, the number of specimens available for such work is extremely limited, but this likely will change with additional exploration of this recently discovered cave system (W. Russell pers. comm.). The Salado population is distinguished from others in the north by a single substitution (which needs to be confirmed by resequencing); however, its high degree of morphological divergence and apparent geographic isolation lead us to believe that it is

a distinct species that simply is not highly divergent for the molecular markers that we have examined from other members of the northern group. The cytb data do provide good evidence of the distinctiveness of the populations in the Lake Georgetown area, mirroring the results of the allozyme studies, in which these populations displayed several unique alleles (see Part I of this report). The Cedar Breaks Hiking Trail Spring population that we used as a representative of this group is characterized by two apparent sequence autapomorphies, four sequence positions that differ from other members of the northern (but not southern) group, and an additional position in which a nucleotide unique in the Texas group (but observed in the outgroup) occurs. Thus we feel confident in recognizing the populations in the vicinity of Lake Georgetown as a distinct species within the northern group, and one that should be treated separately for conservation purposes.

In the above discussion, we have considered the Salado and Lake Georgetown population groups as distinct species, and the cave populations in the north to be of uncertain status based on the molecular data. This leaves the Jollyville Plateau and Round Rock spring populations, which cluster together based on mtDNA and (in some analyses) allozyme data. Together, these could be considered candidates for protection, although given its geographic separation and slight sequence differentiation, the Round Rock population could constitute a separate species. At present, a reasonable conservation strategy might be to target the Jollyville Plateau and Round Rock spring populations and the Travis and Williamson County cave populations together for protection, separately from Salado and Lake Georgetown. This leaves the Bat Well population, which at present is problematic (and the locality is represented by only a single specimen). Further study of salamanders in this area northwest of Georgetown is highly desirable; springs in the Berry Creek drainage (in which Bat Well is located) are particularly likely candidates for new salamander localities in the area.

We have been slow to describe new species in the northern region, because of the above uncertainties. At this point, the most reasonable approach is to describe those groups of populations for which we are confident that we are dealing with distinct species. These are: (1) Salado; (2) the Lake Georgetown area populations; and (3) the Jollyville Plateau spring populations, with tentative inclusion of the Round Rock (Brushy Creek) populations, pending some additional morphometric analyses. Our intent is to formally describe these species and (unless new information comes to light) treat the cave populations in the north as being of uncertain status (although all clearly are part of the northern group). This will help to clarify the uniqueness of the northern populations and formally define some of the component groups that are distinct and should be targeted for conservation efforts. However, additional work, using more variable molecular markers, is crucial to a thorough understanding of relationships and species boundaries in this region, especially with respect to the cave populations. This knowledge is fundamental to development of population-specific conservation strategies for salamanders in this region.

Summary of northern group populations:

**Jollyville Plateau group (Travis Co.; Williamson Co.):**

This is a group of populations associated mainly with the margins of the Jollyville Plateau that shows a high degree of molecular and morphological uniformity and likely represents a single species. Members include the following populations: Balcones Park Spring, Barrow Hollow Springs, Bull Creek Springs, Hanks' Tract Springs, Canyon Creek Springs, Canyon Vista Spring, Horsethief Hollow Spring, Schlumberger Spring, Stillhouse Hollow Springs, Wheelis Tract Springs, and probably the Round Rock (Brushy Creek) populations. Only one Round Rock population is currently known to exist; the other, Krienke Spring, apparently was destroyed by

human activities (Sweet 1978a). The MacDonald Well locality (Sweet 1978a, 1982) probably also represents this species, but we have been unable to obtain specimens from this site for molecular analysis and the population may no longer exist due to human impacts (personal observations). Many new localities are likely to be discovered, especially in the Bull Creek drainage (in the course of this study, we approximately quadrupled the number of known localities in the area). However, all the known localities are vulnerable to damage and degradation of water quality due to increasing development in the region.

**Lake Georgetown area (Williamson Co.):**

Salamanders from springs in the vicinity of Lake Georgetown display a unique combination of alleles that distinguish them from other members of the northern assemblage of populations, specifically unique alleles at medium frequency at the aconitate hydratase 1 and creatine kinase 1 loci and an apparently fixed unique allele at the alpha glycerol-3-phosphate dehydrogenase locus. The mtDNA data (see above) also readily distinguish this group from all others. Preliminary investigations suggest that these salamanders can also be distinguished from other members of the northern assemblage based on characteristics of the lateral iridophore rows. We informally regard members of this group (represented by Avant's, Buford Hollow, Crockett Garden, and Cedar Breaks Hiking Trail Spring, all populations discovered during this study), as a distinct species, and plan to formally describe them. The status and relationships of the population in the riverside springs in the park within the city of Georgetown are uncertain; these springs have been heavily modified and appear unlikely to support a healthy population of salamanders. We collected one tiny juvenile at the middle of the three park springs in 1992, but were unable to resolve the key allozyme loci due to the tiny size of the specimen (mtDNA data may be of help in determining the relationships of this population but are not yet available).

**Salado Springs (Bell Co.):**

Salamanders from these springs are very distinctive morphologically, with elongate bodies, large rectangular heads, uniform brown to gray-brown coloration, and very reduced eyes. Discriminant morphometric analyses (Chippindale et al 1991) readily separate individuals from this population from those from other surface populations. The Salado salamanders also share a peptidase D allele with animals from the Georgetown group, an allele that otherwise appears to be very rare in the northern region. As discussed above, this population is distinguishable from members of the Jollyville Plateau/Round Rock group by only a single cytb sequence substitution that remains to be confirmed, so the mtDNA data add little to our understanding of the affinities of this morphologically distinct and apparently isolated group. Based on the available information (primarily morphology and distribution), we regard the Salado group as a distinct species and plan to describe it as such.

**Kretschmarr Salamander Cave (Travis Co.):**

Located on the Jollyville Plateau, salamanders in this tiny stream cave appear morphologically similar to surface populations in the area. They are distinguished from spring populations in the Jollyville Plateau region allozymically by a high-frequency glucose-6-phosphate isomerase allele that otherwise is rare in the area, and do not cluster with the Jollyville Plateau spring populations based on the mtDNA data. The taxonomic status of this population remains unclear.

**Testudo Tube (Williamson Co.):**

Salamanders in this cave appear morphologically similar to animals from surface populations, unlike individuals from the nearby Buttercup Creek Cave system which show pronounced troglobitic morphologies. Surface populations of *Eurycea* occur in springs on the nearby

Audubon property (Chippindale et al. 1992); however, we have been unable to collect animals from the Audubon localities for comparison to those from Testudo Tube. The mtDNA data place this population as sister to a representative from the Buttercup Creek Cave system (see below).

**Buttercup Creek Cave System (Williamson Co.):**

We have chosen to group together individuals from Buttercup Creek Cave, Twasa Cave, Ilex Cave, and Treehouse Cave, because this series of caves is well defined geographically, apparently all are hydrologically connected (Russell 1993), and adult salamanders from throughout the system show a strong troglobitic morphology (e.g. reduced eyes and pigmentation). There is substantial allozyme variation in this group, and not all members cluster together phenetically. However, sample sizes for each of these caves are very small and thus there is a high probability of sampling (and therefore clustering) error for this group, particularly given the low levels of genetic differentiation that characterize most members of the northern assemblage. Our working hypothesis is that this group represents a distinct species, but at present we are reluctant to formally describe it, pending acquisition of more specimens and examination of rapidly evolving molecular markers. The mtDNA sequence data place the Ilex Cave sample that we examined as sister to the Testudo Tube population, which is not surprising based on geographic proximity.

**Bat Well (Williamson Co.):**

Little is known about salamanders at this locality, and we have only been able to obtain a single specimen whose affinities are unclear. This specimen possessed a Peptidase D allele that also characterizes the Georgetown and Salado population groups, but lacked the aconitate hydratase 1 and creatine kinase 1 alleles that occur at medium frequency in the Lake Georgetown group, as well as the alpha glycerol-3-phosphate dehydrogenase allele that further characterizes members of the Lake Georgetown group. Based on the mtDNA sequence data, this population also does not cluster with the Lake Georgetown group and its taxonomic status is uncertain.

Southern group: distinctive members in the southeastern region

***Eurycea* (formerly *Typhlomolge*) *rathbuni* (Hays Co., subterranean waters at San Marcos):**

This is a distinct species that is readily distinguishable based on morphology and molecular evidence. It appears to restricted to the San Marcos Pool of the Edwards Aquifer. Relationships of the presumed sister taxon *E.* (formerly *T.*) *robusta* are uncertain, due to lack of availability of fresh specimens. We follow Mitchell and Reddell (1965) and Mitchell and Smith (1972) in use of the name *Eurycea rathbuni*, since the molecular evidence indicates that this species is nested phylogenetically within the Texas *Eurycea*. Analysis of the cytb data with equal weighting of transitions and transversions suggests that *E. rathbuni* (presumably together with *E. robusta*, for which we have no data) is the sister taxon to other southern Edwards Plateau *Eurycea*. *E. rathbuni* is characterized by a host of cytb sequence substitutions that are unique (at least in the Texas group; some are shared with members of the northern group and/or outgroup), and this information, together with its unique morphology, allozyme profile, and genome size, strongly supports continued recognition of this salamander as a distinct species.

***Eurycea nana* (Hays Co., San Marcos Springs):**

We discuss the status of this species elsewhere in this report (see discussion of the Comal Springs group) and in others (e.g. Chippindale et al. 1993). It clearly represents a distinct species, restricted to the outflows of San Marcos Springs. Its precise position in the context of

the southern group is uncertain, as its placement differs somewhat based on allozyme versus mtDNA sequence data (see Figures 1, 2, and 3), but several different lines of molecular evidence, together with its unique morphology, support recognition of this as a highly distinct species. The cytb data support our earlier conclusion that *E. nana* is restricted to San Marcos Springs and clearly does not include the population at Comal Springs; San Marcos *E. nana* are characterized by at least two sequence autapomorphies.

**Pedernales group (Travis Co.):**

This group was discovered in the course of this study, and the full extent of its distribution is unknown. It appears to be geographically strongly isolated from other populations of *Eurycea*. The two known localities are springs adjacent to the Pedernales River directly across from Westcave Preserve; we have searched springs and caves on the Preserve for salamanders with no success. These are small salamanders, and in these populations a distinctive "golden" color morph is common. Apparently unique alleles at the Ldh-A and Mdhp loci occur at medium frequency. The cytb sequence data confirm the distinctiveness of the Pedernales populations (although as noted in Methods, it has not been possible to obtain complete sequence despite repeated attempts). Like *E. nana*, although the precise placement of these populations in the southern group is uncertain, it appears from the available evidence that they represent a distinct and divergent species.

Other populations and taxa in the southeastern region

Based on the cytb data, the southeastern group as a whole is readily distinguishable from members of both the northern group (see above) and the southwestern group, from which members of this group are differentiated by at least seven cytb sequence differences. Of these, two are shared with *E. rathbuni*, one excludes *E. nana*, and one constitutes an unambiguous synapomorphy when character state distributions in the outgroup are considered. Although the cytb sequence data shed little light on relationships or species boundaries in the southeastern group exclusive of *E.nana* and the Pedernales populations, allozymes and in some cases morphology have allowed us to recognize some additional major divisions in the group. *E. sosorum* and *E. tridentifera* remain readily distinguishable as separate species based on morphology and allozymes (and geographic distribution in the case of *E. sosorum*); the *E. pterophila* (Blanco River drainage) and *E. neotenes* (Helotes and area) groups are distinguishable based on allele frequency patterns and geographic distribution; and relationships of the remainder of the group (which we very tentatively designated the "*latitans*" group in Part I of this report) remain uncertain. Topotypical *E. latitans* are very unlikely to represent hybrids between *E. tridentifera* and surface populations (as suggested by Sweet 1984) based on the allozyme data (see below). Members of the southeastern group would be excellent candidates for detailed investigations of relationships and species boundaries using very rapidly-evolving molecular markers (see Conclusions and Recommendations).

Summary of southeastern group members

***Eurycea sosorum* (Travis Co.; Barton Springs ):**

Salamanders from Barton Springs clearly represent a distinct species, and this species recently was formally described; details were provided by Chippindale et al. (1993). Like many other members of the southeastern group (including the otherwise distinct species *E. tridentifera*) this species is not differentiable from others in the region based on the cytb data.

***Eurycea pterophila* group (Blanco, Hays, and Kendall Counties; Blanco River drainage):**

This group of populations shows a relatively high degree of cohesiveness based on allozyme data and geographic distribution, and we will formally resurrect the name *E. pterophila* (formerly applied to the Fern Bank population), which Sweet (1978b) regarded as invalid. This species is not distinguishable from others in the southeastern region based on cytb sequences. Members of this group include populations at Fern Bank Springs, Peavey's Springs, Boardhouse Springs, Zercher Spring, Grapevine Cave, and T Cave.

**Comal Springs (Comal Co.):**

Sweet (1978) suggested that the population at Comal Springs might represent *E. nana*, but allozyme evidence (Chippindale et al. 1993), cytb sequence data (this report) and morphometric analyses (Chippindale et al., unpublished) indicate that the populations at San Marcos and Comal Springs are readily distinguishable from one another and clearly are not conspecific. The Comal Springs population appears to be geographically isolated from others; morphologically these salamanders are similar to those from many other spring populations. Allozymically, Comal Springs salamanders share a medium-frequency aconitase-1 allele that otherwise has been detected only in *Eurycea rathbuni*. There is one potential mitochondrial sequence autapomorphy (a G-A transition), but this is somewhat ambiguous and remains to be confirmed. Clearly, further study of the Comal Springs population and others in the southeastern region is necessary; presumably very rapidly-evolving molecular markers will be necessary to do this effectively. At this point, we regard the Comal Springs population as a distinct species based on an evolutionary species concept, but one which has undergone little differentiation based on the markers that we have examined so far.

**"*Eurycea latitans* group " (Comal, Kendall, and Hays Counties):**

This is one of the most heterogeneous groups that we informally recognize here, and includes the following populations: Pfeiffer's Water Cave, Bear Creek Springs, Cibolo Creek Tributary Spring, Kneedeep Cave Spring, Honey Creek Cave Spring, Less Ranch Spring, Cherry Creek Spring, Cloud Hollow Springs, and Rebecca Creek Spring. This is largely a grouping of convenience, based on overall similarity in gene frequencies, and may contain multiple species. We recognize this group as the *latitans* group because this name is available; Sweet (1978, 1984) regarded the name as invalid because he believed *E. latitans* to be hybrids between *E. neotenes* and *E. tridentifera*. However, in an allozyme survey that included five individuals from Pfeiffer's Water Cave (adjacent and hydrologically connected to the type locality for *E. latitans*, Cascade Caverns), we found these salamanders markedly different in allele frequencies from *E. tridentifera* from three different localities (Honey Creek Cave, Ebert Cave, and Badweather Pit). In particular, the *latitans* lacked a diagnostic NADP-dependent malate dehydrogenase allele that appears to be fixed or near-fixed in populations of *E. tridentifera*. Thus, it seems unlikely that this population is a hybrid swarm and (since it also does not appear to represent *E. neotenes*) the only logical solution is to reinstate the name *E. latitans*, at least for this population and possibly for others in the area. For this reason, and because species boundaries in the "*latitans*" group remain poorly understood, it would be inadvisable to remove this taxon from listing at present, as has sometimes been suggested based on the hybridization hypothesis. The cytb data provide no new information on relationships within this putative group.

***Eurycea tridentifera* group (Comal and Bexar Counties; Kendall Co.?):**

This group includes morphologically specialized troglobites that form a fairly homogeneous group based on morphometric analyses (Sweet 1978a, 1984). Allozyme evidence for individuals from three populations (Honey Creek Cave, Ebert Cave, and Badweather Pit) supports Sweet's conclusion that this is a genetically relatively cohesive group and is likely to represent a single

species. The cytb data do not contradict this hypothesis, except that a single transition was detected for the representative from the Badweather Pit population, suggesting some molecular divergence in the group. Refer to Sweet (1977a) and Chippindale et al. (1993) for additional localities at which *E. tridentifera* is thought to occur.

***Eurycea neotenes* group (Bexar Co.):**

Members of the Helotes Creek Spring, Leon Springs, and Mueller's Spring populations cluster together based on similarities in allele frequencies, and are distinguished from other populations in part by rare alleles at the glucose-6-phosphate isomerase and phosphoglucomutase loci. Since the Helotes Creek Spring site represents the type locality for *E. neotenes* and this group forms a well-defined geographic assemblage, we regard the members of this group as the only "true" *Eurycea neotenes*. Based on the evidence thus far, application of this name to other central Texas *Eurycea* is inappropriate, especially since other named species appear to cluster phylogenetically within the group formerly assigned to *E. neotenes*. This species is not distinguishable from others in the southeastern region based on cytb sequences.

## Southwestern group

While no unambiguous synapororphies exist for the southwestern group, there are at least seven sequence differences that distinguish members of this group from the southeastern group (all are either shared with members of the northern group and/or *E. rathbuni*, or with members of the outgroup). The apparent "break" between populations in the southeastern and southwestern groups corresponds precisely to the geographic division that we found in allozyme allele frequencies, although the exact location of the break remains uncertain (we have narrowed the geographic "gap" but not filled it completely, so the possibility that a narrow cline exists remains). Within the southwestern group there is substantial differentiation: the morphologically distinct Carson Cave population (previously suspected to represent a distinct species; J. Reddell pers. comm.) is quite distinct fom others and appears as basal to the rest of the southwestern group; the Camp Mystic population (which is quite distinct based on allozymes) also possesses one autamorphy and another sequence difference that is unique in the southern region; and the morphologically distinct Tucker Hollow Cave population (previously suspected to represent a distinct species; J. Reddell pers. comm.) is characterized by one sequence difference that apparently is unique in the Texas group. Thus it appears that multiple species may be present in the region. The cytb sequence data indicate that our earlier "Carson Cave" grouping probably was overly inclusive (as we suspected it might be) and as with the other major population groups that we have discussed, additional study using more rapidly evolving molecular markers would be valuable.

The cytb sequence data for *E. troglodytes* from Valdina Farms Sinkhole (which may now be extinct) place it squarely within the southwestern group and provide no support for Sweet's (1984) hypothesis that this population represented hybrids between *E. tridentifera* and surface populations. Given this situation, there is no reason to suppress the name, and it should be resurrected for this population and perhaps for others in the southwestern region.

### Summary of southwestern group members

**Camp Mystic (=Edmunson Creek Spring; Kerr Co):**

Animals from this locality are characterized by unique, apparently fixed alleles at the malate dehydrogenase 1 and pyruvate kinase loci, and thus are distinct genetically from other populations that we have examined. As noted above, they also can be distinguished based on cytb sequence markers. Morphologically they appear superficially similar to individuals from

other populations in the region, and the taxonomic status of this population is uncertain. Additional sampling in the area may help to shed light on the status and distribution of this apparently distinct lineage.

**176 Spring (Kerr Co.):**

We chose to separate this population from others due to a moderate degree of genetic differentiation from other populations in the area, primarily at the alpha glycerol-3-phosphate locus. The taxonomic status of this population is uncertain, and sequence data are incomplete; clearly it is a member of the southwestern group but at present its affinities are uncertain.

**Greenwood Valley Ranch Springs (Real Co.):**

These three springs are near the northwestern edge of known range of *Eurycea* in the Edwards Plateau region. Salamanders from this area are characterized by a distinct allele at the isocitrate dehydrogenase 1 locus and lack of activity at the malate dehydrogenase-2 locus, and thus are distinct genetically from the other populations that we have examined. The taxonomic status of this group is uncertain, but based on the cytb data it clearly is part of the southwestern group.

**Sabinal River Springs (Bandera Co.):**

Salamanders from the two springs placed in this grouping, Sabinal Canyon Spring and Murphy's Spring, are characterized primarily by an otherwise rare allele at the NADP-dependent malate dehydrogenase locus. Salamanders from one of these localities (Murphy's Spring) are known to undergo natural metamorphosis (Sweet 1977b). The taxonomic status of this group is uncertain but based on the cytb data it clearly is part of the southwestern group.

**Tucker Hollow Cave (Real Co.):**

Salamanders in this tiny cave exhibit a distinctive morphology similar in some respects to that of individuals from the Carson Cave population (see Sweet 1978, 1984 for details of morphometric analyses). Individuals from this locality also possess a characteristic allele at the isocitrate dehydrogenase 1 locus and a single, apparently unique sequence substitution. Thus, this population probably represents a distinct species.

***E. troglodytes* (Valdina Farms Sinkhole, Medina Co.)**

As noted above, the sequence data for the (likely extinct) population formerly known as *E. troglodytes* do not support Sweet's (1978, 1984) hypothesis that it is a hybrid between surface populations and *E. tridentifera* (a species known only from a geographically distant area of the southeastern region). Instead, the new cytb data place this population where it would be expected based on distribution and biogeographic considerations, consistent with the pattern of relationships revealed by molecular data for most other central Texas *Eurycea*. One could argue that the mtDNA data reflect only female-mediated patterns of inheritance, and thus conceivably *E. tridentifera* nuclear genes (and/or mitochondrial haplotypes) could also be present in the population. However, this seems unlikely, especially given the lack of evidence of a hybrid origin even for *E. latitans*. Since *E. troglodytes* appears to be a member of the apparently monophyletic southwestern group and does not represent an occurrence of either *E. neotenes* or *E. tridentifera*, the name must be resurrected and applied at least to animals from this locality. The name may also apply to members of what we initially called the "Carson Cave group" (see below) and others, but additional study is necessary to clarify this issue.

**"Carson Cave group" (Edwards, Gillespie, Kerr, and Uvalde Counties):**

Like the "*latitans* group", this is a heterogeneous assemblage of populations that we grouped together based primarily on similarity in allele frequencies in Part I of this report. More than one species may be involved, and this possibility is supported by the subdivision of this group provided by the cytb sequence data. In Part I of this report, we included in this group the following localities: Carson Cave, West Nueces Spring, Sutherland Hollow Spring, Dutch Creek Spring, Robinson Creek Spring, Wetback Spring, Trough Spring, and Fessenden Springs. Individuals from the Carson Cave population are very large and exhibit a troglobitic morphology distinct from other members of this group; this population has sometimes been regarded as a distinct species based on its morphology (J. Reddell, personal communication). Sequence data also reveal the distinctiveness of this population, although study of additional populations from this western area is desirable (and is in progress). The cytb data place the Trough Spring population as sister to the Camp Mystic population, which makes sense in terms of the geographic proximity of these populations. Similarly, the Sabinal populations are placed by analyses of the sequence data in a group that includes Sutherland Hollow and *E. troglodytes*, a biogeographically logical grouping (see above). Members of the Sabinal and Sutherland Hollow populations are known to undergo natural metamorphosis (Sweet 1978), which also hints at a close relationship. Thus in the case of the southwestern group, the cytb data appear to provide a basis for partitioning that is more consistent with the biogeography of the area than the arrangement suggested by the allozyme data alone. However, additional investigation of the southwestern group is necessary to more fully determine the status of its component populations.

## CONCLUSIONS AND RECOMMENDATIONS

The central Texas *Eurycea* are a highly diverse group, and occupy what are, in effect, "islands" of spring and cave habitat in the Edwards Plateau region. This situation, coupled with large-scale biogeographic and geological factors in the history of the group, has led to a high degree of differentiation in many cases, and the formation of what we believe to be many distinct species. Specific recommendations for treatment of each of the species and major population groups that we recognize are contained in the preceding sections. In the primarily molecular work that we have conducted, we have identified the major biogeographic groupings in the central Texas *Eurycea* and made reasonable progress toward an understanding of differentiation and species boundaries on a fine scale. The mitochondrial sequence data presented here have contributed to this knowledge, but it also has become clear that many different approaches will be necessary to unravel the complexities in this assemblage. One very promising direction for future research is the use of rapidly-evolving markers to investigate differentiation in some of the problematic areas (especially the northern and southeastern groups). Sequences of the mitochondrial D-loop (control region) would be particularly good candidates, as would sequences from nuclear introns (Larson and Chippindale 1993). Palumbi and Baker (1994) provided sequences for intron primers and outlined strategies for investigation of such genomic regions. The central Texas salamanders appear ideal for application of these new and potentially very powerful techniques for detection of genetic variation, and we strongly recommend that this direction be pursued. This work is especially important because habitats in some of the regions of interest are subject to intense development pressure, and therefore a fine-scale understanding of population relationships will be essential to the development of effective conservation strategies.

In this report we have identified major population groups and species in the central Texas *Eurycea*, discussed differentiation among these groups, and identified those units that are particularly divergent based on the available molecular evidence. This information provides the

basis for regional conservation strategies for protection of different members of the central Texas *Eurycea* assemblage. However, effective conservation strategies should focus not just on the salamanders, but also the aquatic systems in which they live. Given the degree of differentiation that study of the salamanders has revealed, it seems likely that there are other aquatic organisms in the region that might reveal similar patterns of divergence. Thus many aquatic species of which we are not even aware may share spring and cave habitats with the salamanders. These ecosystems are particularly vulnerable to destruction due to factors such as pollution or depletion of the aquifers that supply them. Thus, every effort should be made to preserve the quality and quantity of water in the springs and caves of the Edwards Plateau region, to protect not only the many unique and endemic salamanders in the region, but also the unique and complex ecosystems that these waters support.

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# FIGURE LEGENDS

Figure 1: Phenetic clustering of populations of Texas *Eurycea*, based on allozyme electrophoresis of the products of 25 enzyme-encoding loci. Informal groups that we recognize are indicated in capital letters; the Buttercup Creek Cave group contains Buttercup Creek, Ilex, Treehouse, and Twasa Caves. See text for additional details of this analysis. Population groupings shown here are those we designated in Part I of this report, before the mtDNA data were available.

Figure 2: Phylogenetic (parsimony) analysis of working groups of Texas *Eurycea*, using frequency-based coding of allelic composition. Refer to text for additional details of this analysis. Note that the rooting (which renders the northern group paraphyletic) is questionable (see text for discussion).

Figure 3: Strict consensus of 288 equally most parsimonious trees based on the mitochondrial cytochrome b sequence data, with transitions and transversions weighted equally. See text for details of the analysis and composition of the outgroup (not shown here).

Figure 4: Sample parsimony tree from partial heuristic search for all populations and taxa. This is a phylogram, i.e. branch lengths are proportional to the number of evolutionary steps required by this arrangement. Thus, branch lengths shown here give a rough indication of the degree of cytochrome b sequence divergence for each population or population group. Note that this is only one of many different possible treees from an incomplete analysis and thus is NOT intended to represent the best estimate of phylogeny. See text for further explanation.

Figure 1, part 1 (phenetic clustering of northern populations)

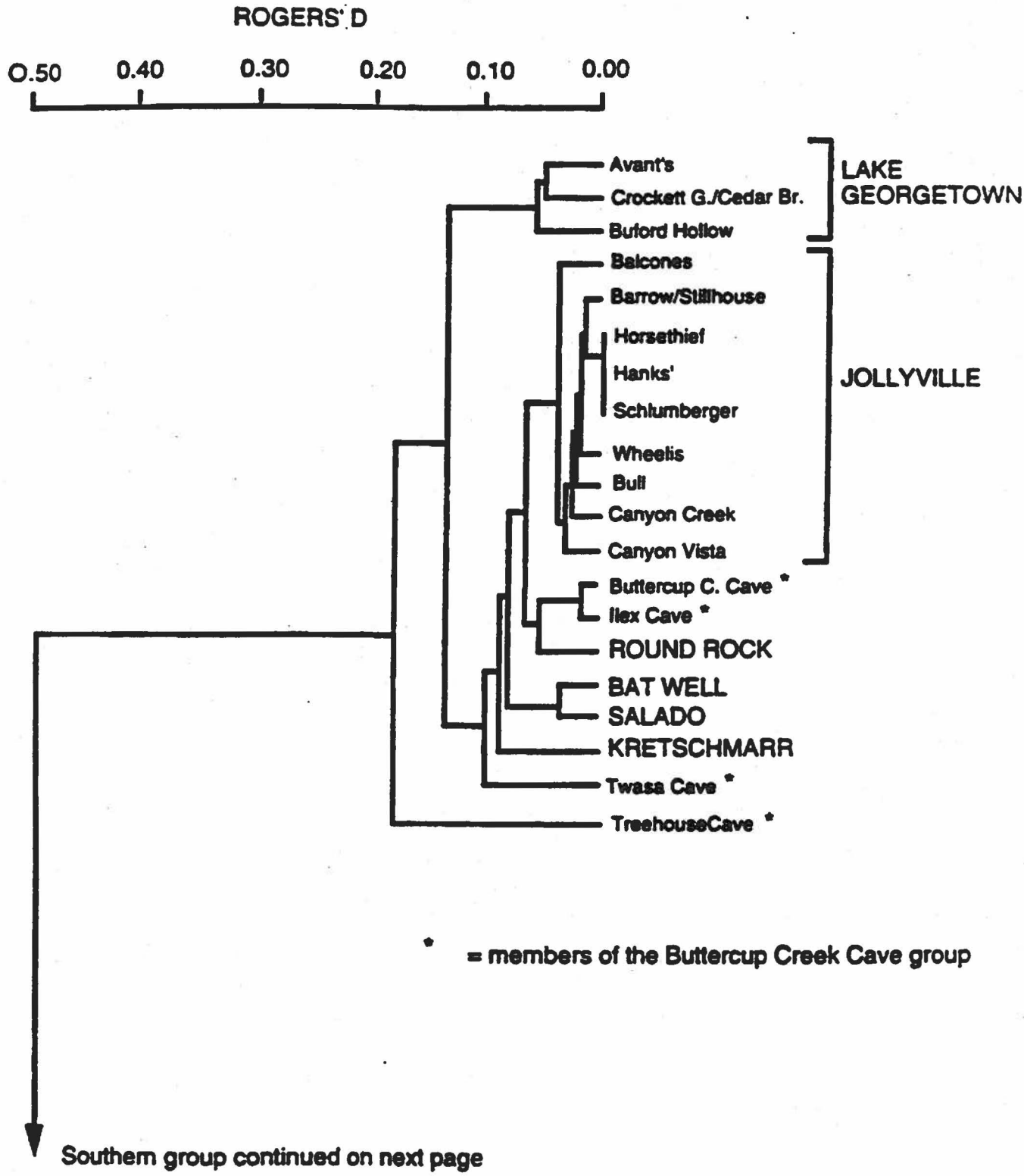


Figure 2

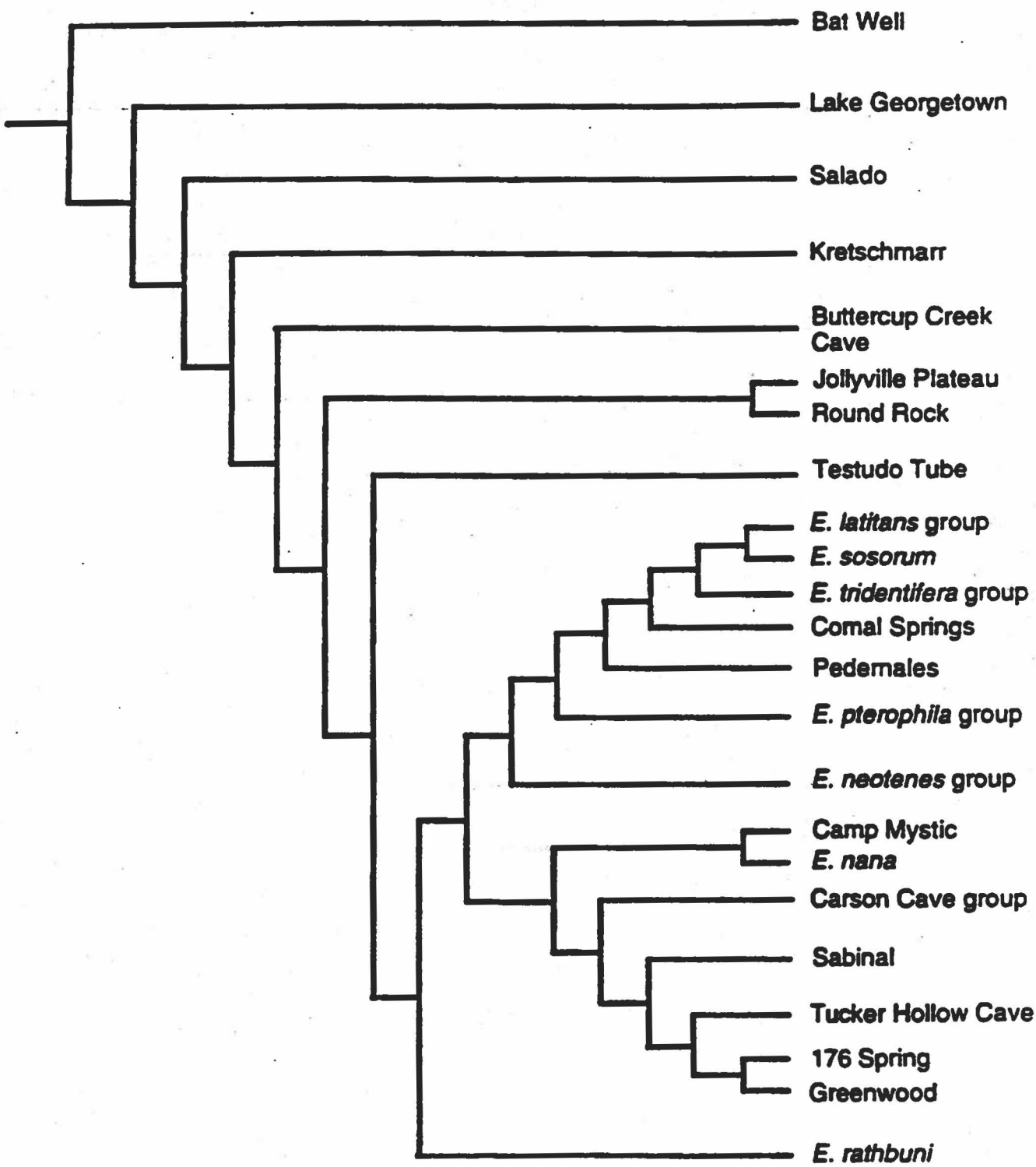
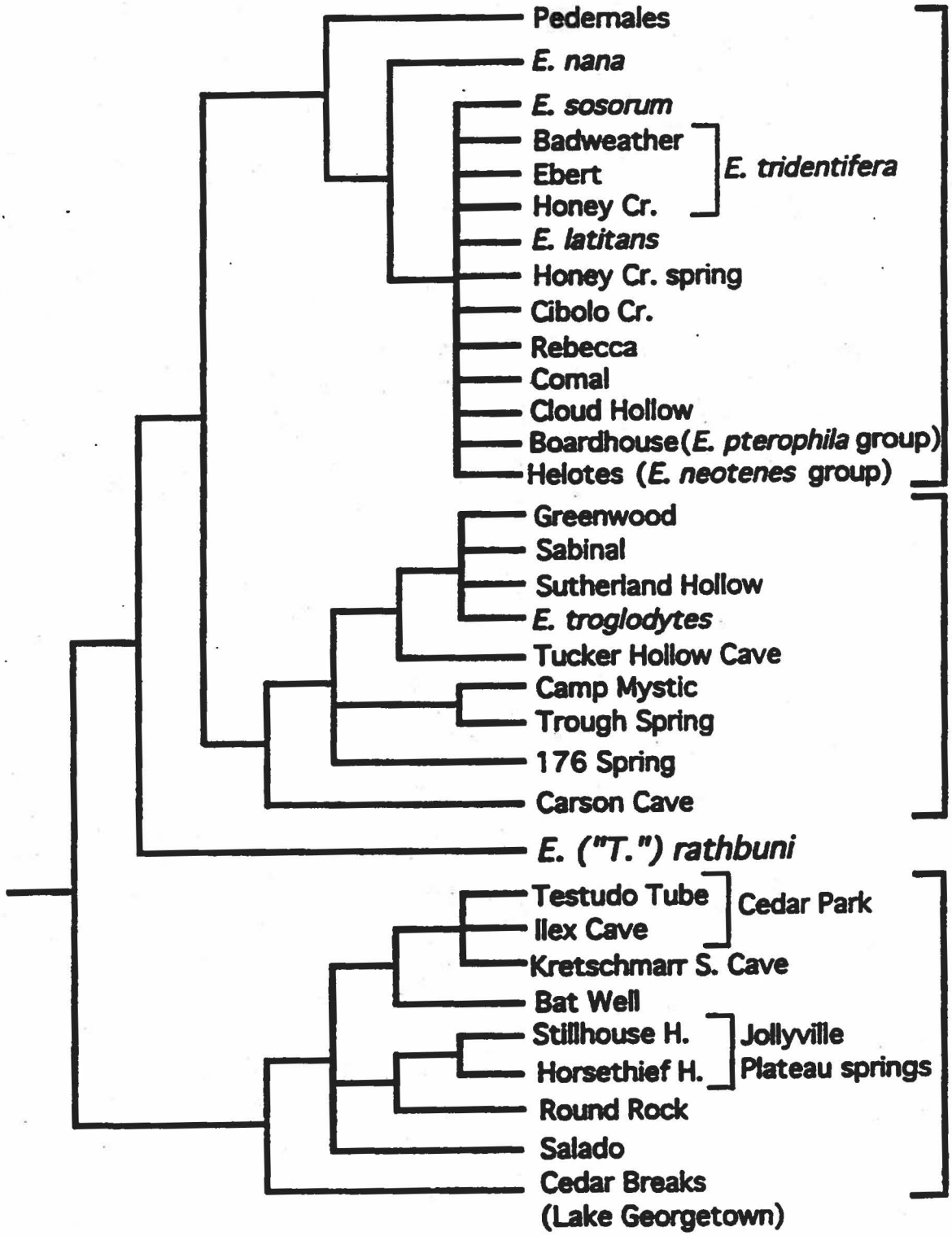

Bat Well
Lake Georgetown
Salado
Kretschmarr
Buttercup Creek Cave
Jollyville Plateau
Round Rock
Testudo Tube
E. latitans group
E. sosorum
E. tridentifera group
Comal Springs
Pedernales
E. pterophila group
E. neotenes group
Camp Mystic
E. nana
Carson Cave group
Sabinal
Tucker Hollow Cave
176 Spring
Greenwood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

Figure 3


Pedernales
E. nana
E. sosorum
Badweather
Ebert
Honey Cr.
E. tridentifera
E. latitans
Honey Cr. spring
Cibolo Cr.
Rebecca
Comal
Cloud Hollow
Boardhouse (E. pterophila group)
Helotes (E. neotenes group)
SOUTHEAST
Greenwood
Sabinal
Sutherland Hollow
E. troglodytes
Tucker Hollow Cave
Camp Mystic
Trough Spring
176 Spring
Carson Cave
SOUTHWEST
E. ("T.") rathbuni
Testudo Tube
Ilex Cave
Cedar Park
Kretschmarr S. Cave
Bat Well
Stillhouse H.
Horsethief H.
Jollyville Plateau springs
Round Rock
Salado
Cedar Breaks
(Lake Georgetown)
NORTH