FINAL REPORT

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PROGRAM TITLE: Endangered and Threatened Species Conservation

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PROJECT NUMBER: 3.4

PROJECT TITLE: Status of Central Texas Salamanders (Genus: *Eurycea*)

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).

ACTUAL SEGMENT COSTS: $ 0.00

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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).

<u>Part I</u> 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.

<u>Part II</u> 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.

<u>Part III</u> 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. ~~125;~~ 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.

Ref. 102

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*.

Southwestern groups

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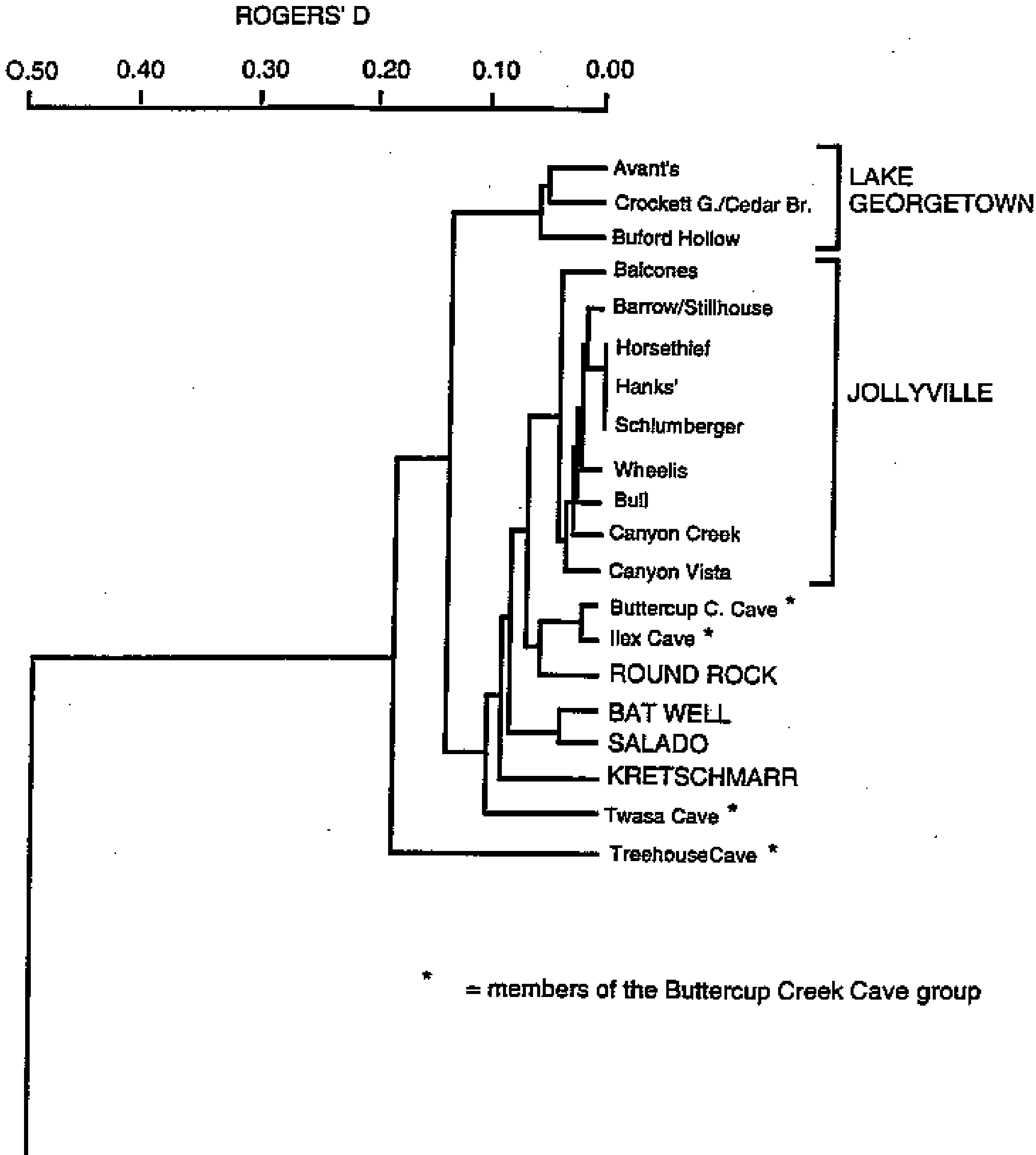
Figure 1, part 1 (phenetic clustering of northern populations)



Southern group continued on next page

Figure 1 continued

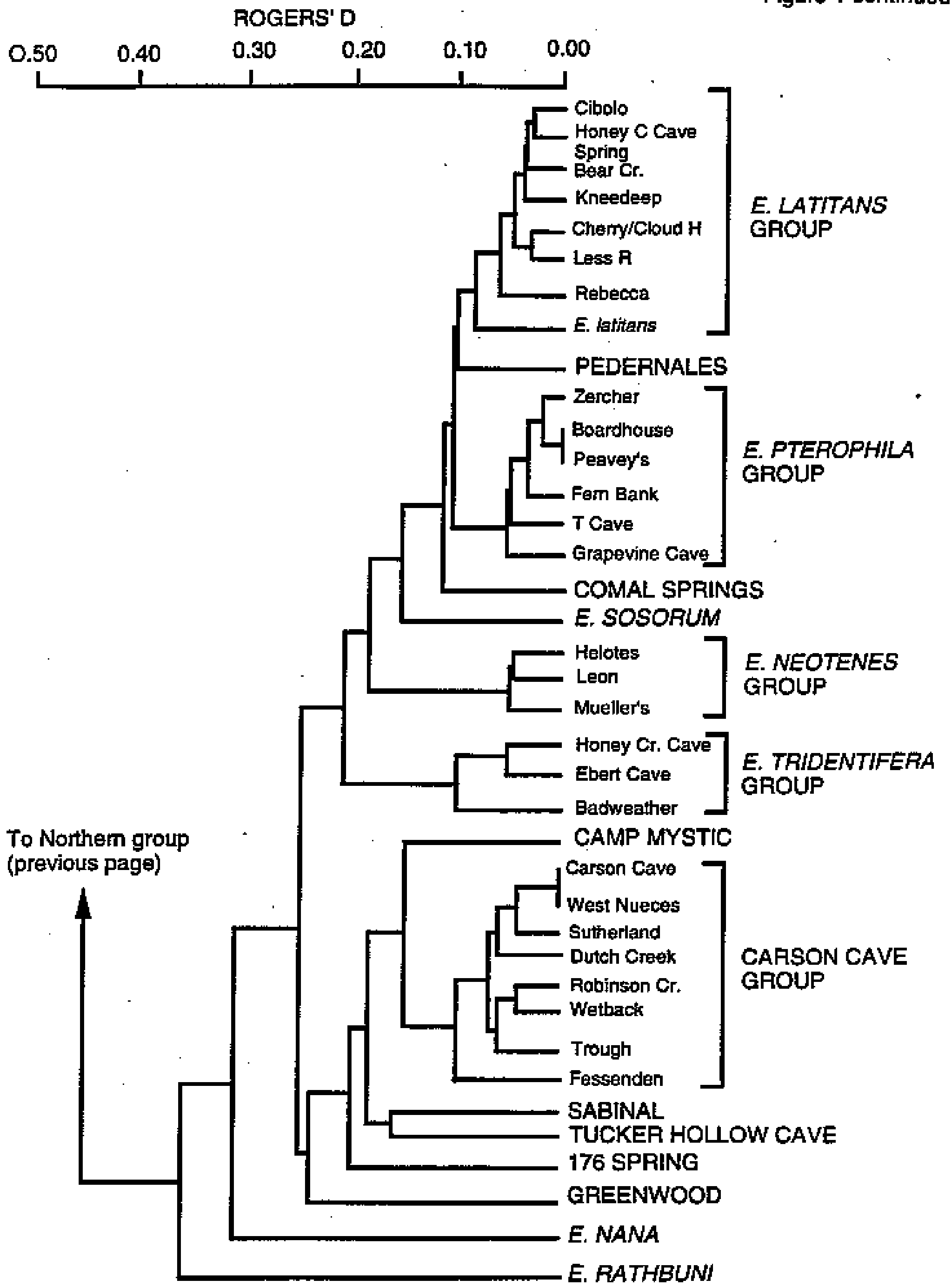
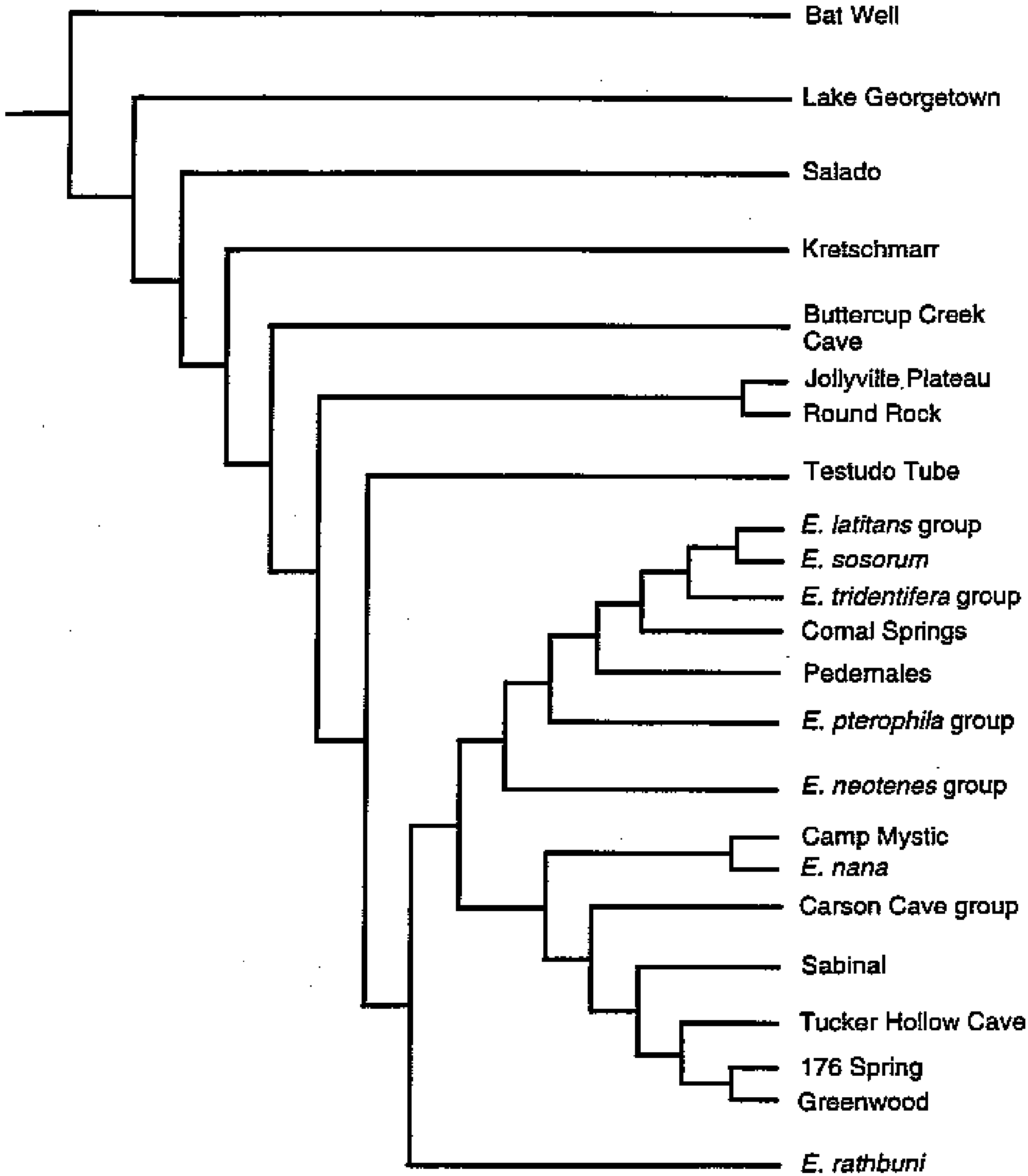


Figure 2



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.

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).

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.

<u>Populations included</u>:

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.

<u>Summary of northern group populations:</u>

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 I 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.

<u>Southern group: distinctive members in the southeastern region</u>

***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.

<u>Other populations and taxa in the southeastern region</u>

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).

<u>Summary of southeastern group members</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). 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.

<u>Southwestern group</u>

While no unambiguous synaporomphies 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 automorphy 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.

<u>Summary of southwestern group members</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. 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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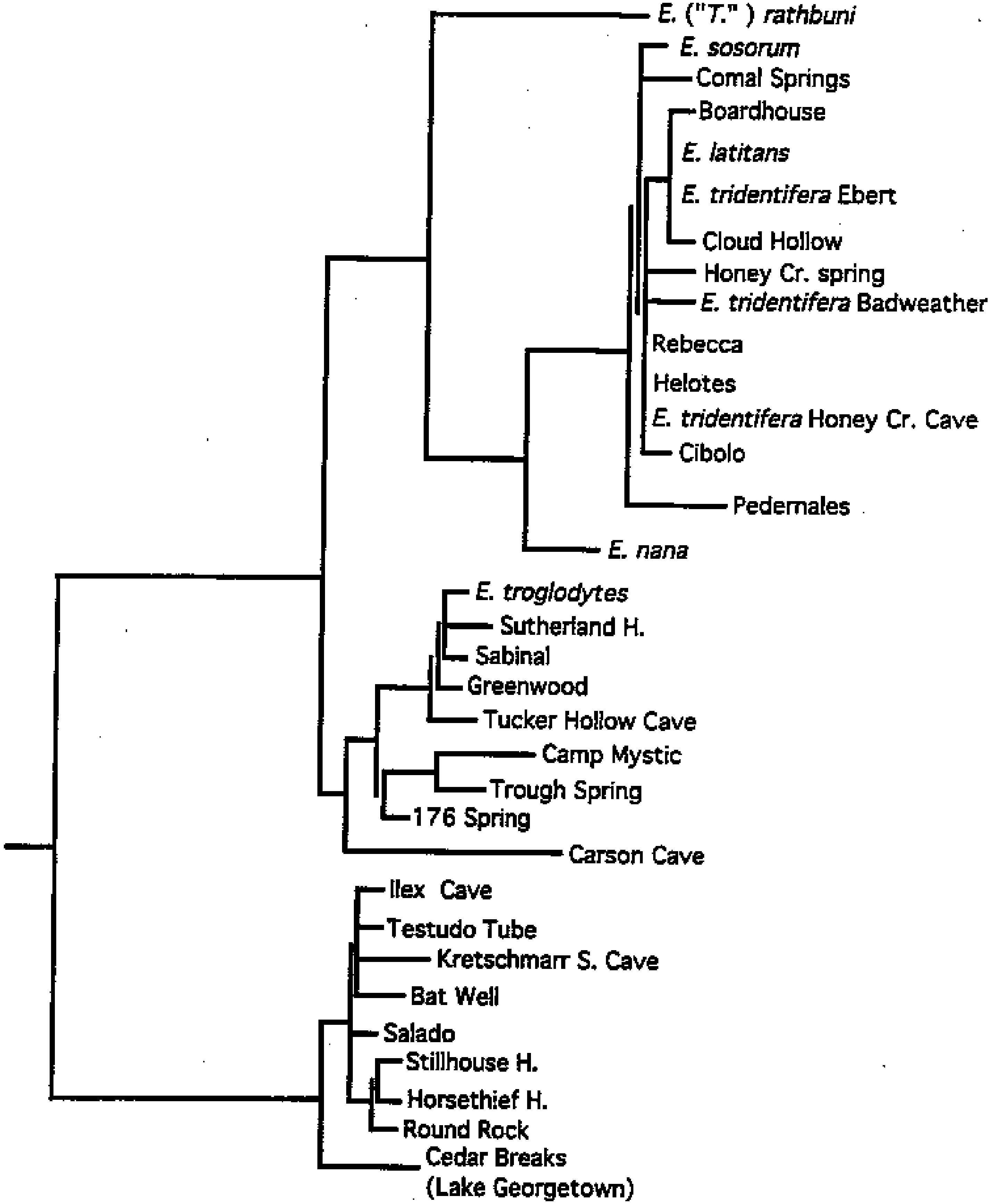
Figure 4



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

A Status Report on the Threats Facing Populations of Perennibranchiate Hemidactyliine Plethodontid Salamanders of the Genus *Eurycea* North of the Colorado River in Texas.

Final Section 6 Report, Part III

Edited by G. A. Rowell

Wildlife Diversity Program
Texas Parks & Wildlife Department
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Biological Background

Central Texas hemidactyliine plethodontid salamanders belonging to the genera *Eurycea* and *Typhlomolge* have been a focus of study for some time (10-15,71,72). This distinctive group of salamanders is endemic to the Edwards Plateau physiographic region, occurring in 16 counties ranging from Bell County in the north to Val Verde County in the west (Figures 1 and 2). They have obligately aquatic eggs and larvae, and the adults of almost all populations studied retain the larval morphology (*neoteny*). These animals are physiologically adapted to and inhabit thermally constant spring complexes and water-bearing cave systems and appear to require specific water quality and/or quantity characteristics of these habitats to successfully complete aspects of their life-cycle (10-15,71,72). For instance, gravid salamanders of the *Eurycea neotenes* complex do not oviposit when held in aquaria with ordinary filtered tap water (15). It has been demonstrated that flowing spring water and physical cues mimicking the conduits of natural springs are required to elicit proper oviposition behavior (55). These salamanders feed on a wide variety of small invertebrates such as amphipods which are also dependent on these aquatic habitats.

The species which has been known as *Eurycea neotenes* is actually a polyphyletic grouping of several species (10-15). In particular, the populations north of the Colorado River in Travis, Williamson and Bell counties represent a distinctive clade within the *Eurycea neotenes* group. The 3 new species from this area are:

The Jollyville Plateau Salamander (*Eurycea* sp. 1). This species is endemic to springs and caves of the Bull Creek, Cypress Creek, Long Hollow Creek, and Walnut Creek drainages in Travis County (10-14, Figure 3). Most of the known range of this species occurs on a single USGS 7.5' quadrangle (Jollyville), approximately 50 square miles. There is also an enigmatic population of salamanders in a spring along Brushy Creek just east of Round Rock in Williamson County tentatively assigned to this species (13-14).

The Georgetown Salamander (*Eurycea* sp. 5). This species is endemic to springs and caves in the San Gabriel River, Berry Creek, and Cowan Creek drainages west of the city of Georgetown in Williamson County (10-14,89, Figure 3). The areal extent of the known range is less than that of the Jollyville Plateau Salamander, occurring on the western half of the Georgetown 7.5' quad-rangle and along the eastern edge of the adjacent Leander NE 7.5' quadrangle.

The Salado Springs Salamander (*Eurycea* sp. 2). This species is endemic to the group of springs collectively known as Salado Springs (6,10-14, Figure 3) along Salado Creek in and near the town of Salado in Bell County. Specimens have been taken only from the outlets known as Robertson Springs and Big Boiling Springs.

Many of the cave populations in this area are enigmatic, particularly those associated with the subterranean Buttercup Creek stream system near the town of Cedar Park in Williamson

County. The few specimens available from this system are highly divergent morphologically. Not enough material exists to determine their relationship, although they clearly belong to the distinctive northern clade discussed herein. They are regarded taxonomically as of uncertain status pending future work.

Salamanders have often been observed as far as 1200 m from spring outlets along the headwater tributaries of Bull Creek, Stillhouse and Barrow Hollows, and Long Hollow Creek (10-15, T. Schumann, pers. comm.). This phenomenon is probably common wherever spring influences along streams have not been interrupted by clearing of riparian vegetation or physical barriers such as impoundments or low-water dams, and where predatory fish do not occur or have not been established. Salamanders, including juveniles, are often abundant in stream segments that are intermittently dry (A.H. Price, pers. comm., 90). Salamanders either retreat to nearby springheads and springflows during these periods, or aestivate in suitable interstitial spaces in the substrate until water flow resumes (10). These "boom-and-bust" cycles are most likely a normal component of the life history of these species (27). Therefore the intermittent sustained flows which naturally occur in these streams following rainfall events are likely critical physical features to which these animals are adapted. These flows, in addition to providing nutrient input and the requisite physical flow characteristics (91), also prevent substrate cementation by travertine/marl deposits, common in these streams from rapid chemical precipitation of supersaturated $CaCO_2$ due to turbulence and outgassing (25).

Geological Background

The Jollyville Plateau is a remnant outlier of the Edwards Plateau. Its areal extent (roughly 400 square miles in the 3 counties of interest, Figure 4) and relative flatness occur because the Edwards Limestone in the region remains relatively unfaulted (3,86). The plateau is a karst upland which straddles two major drainages, the Colorado River to the south and the Brazos River to the north (33), and its topography reflects ongoing geomorphic processes associated with this geographic placement. The Travis-Williamson county line approximates this drainage divide. Apparent eastward movement of the Colorado River itself has occurred over geologic time, resulting in close proximity of the main channel to the drainage divide and deep incision of the southern margin of the plateau from capture of streams originally flowing towards the Brazos, such as Bull Creek, and other normal fluvial erosional processes. North of the drainage divide, topographic relief trends much more gradually towards the north and east, and the southernmost tributaries of the Brazos system, such as Brushy Creek and the San Gabriel River, form low-gradient drainageways conforming with the landscape.

Groundwater movement in this area is controlled by surface topography. The Edwards Limestone underlying this region is relatively thin and the groundwater aquifer shallow (91). Reliable water occurs from the Edwards Aquifer at shallow depths or from the underlying Trinity Aquifer at depths of 1200-1400 feet (1-2,62). The Edwards aquifer is 225-450 feet thick north of the Colorado River, thinning from south to north, and may be 50 feet or less along the western edge where maximal surface erosion has occurred (1-3). The area where the Edwards and Georgetown limestones are exposed at the surface is called the "unconfined portion" (33) or the "water-table zone" (67) of the Edwards Aquifer. The hydrologic gradients west of the main faults in the eastern part of this zone are steep, and regional ground-water flow is characterized by a relatively fast flow-through system, estimated between 33-328 feet/year (75). This area is rich in springs (6,33,67) which flow from the base of the Edwards Formation where it contacts the underlying, relatively impermeable, Walnut Formation, as well as from faults and fractures in the Edwards itself. Natural water chemistry of these springs is predominately calcium bicarbonate with up to 50% magnesium and is characterized by relatively low conductance values. Those that continue to flow during drought conditions may be receiving minimal additional recharge from the underlying Trinity aquifer (67).

Each small tributary draining the southern margin of the Jollyville Plateau independently recharges its own groundwater system; there are no distinct contributing zones upstream (7,33). For instance, the recharge area for Bull Creek is essentially limited to its drainage basin of about 22 square miles, roughly bounded by U.S. Hwy. 183, FM 620 and RR 2222 (30,50, P. Bennett, pers. comm., Figure 5). The three main headwater tributaries of Bull Creek have 23 known spring outlets, and springs and seeps occur along its course where the suture between the Edwards and the Walnut Formation is exposed. Only about 15% of incident rainfall is likely to contribute to streamflow and recharge (86). Infiltration of surface water is localized and diffuse, occurring via numerous cracks, crevices and sinkholes in the karst terrain. North of the drainage divide along the Travis-Williamson county line stream-bottom recharge is probably more important along major tributaries like Salado Creek and the north and south forks of the San Gabriel River (6,86). Major faults in the Balcones Fault Zone have bisected these streams and transected the underlying Edwards Limestone, dividing the aquifer into discrete segments and channeling groundwater towards major discharge points to the northeast such as the springs in Round Rock, Georgetown and Salado. Smaller springs discharge upstream again where the suture between the base of the Edwards and the underlying, less permeable Walnut Formation is locally exposed.

The total area that is occupied by the outcrop (recharge) of the Edwards aquifer north of the Colorado River is approximately 400 square miles (3), generally west of I-35. The karst limestones comprising it dip to the southeast and come to lie 1500 ft. below the surface at the maximum extent. Many of the tributaries which support known salamander springs, such as Bull Creek and Cypress Creek in Travis County and the North and South Forks of the San Gabriel River in Williamson County, have cut completely through the Edwards and into the underlying strata of the Walnut and Glen Rose Formations (16,67,86).

Geohydrological studies of the Bull Creek and Cypress Creek watersheds in Travis County and the Salado Creek watershed in Bell/Williamson County have been accomplished, but the results are not available (P. Bennett, pers. comm.).

Assessment of Threats

No attempt is made here to evaluate the degree of protection provided by recent regulations of the Texas Natural Resources Conservation Commission because funding for this project expired and because such an evaluation would be more rigorously undertaken by the agency charged with regulating water quality. Local groundwater characteristics are determined by locally interconnected hydrological and geochemical cycles (5,22,68). Urbanization, concomitant with deforestation, overgrazing, improper soil conservation practices, and poor urban planning, can interfere with the hydrological cycle and reduce recharge to the system by increasing the rate and velocity of runoff and promoting increased levels of withdrawal of groundwater (5,43,44,87). As a result, more water is discharged more rapidly as stream flow from a drainage basin, and there is less recharge and storage in local groundwater reservoirs; springs and spring runs dry up more frequently (21,22). In addition, urban development on karst terrain poses unique and serious threats to local groundwater quality because pollutants have easy access to subsurface water, and water can flow relatively rapidly through the system to distant discharge points such as springs (7,16,19,22,56,77). There may be little chance for rehabilitation of polluted water in such circumstances except for a small amount of dilution. Pollutants may also be stored temporarily in the epikarstic zone of an aquifer only to be flushed by episodic flood events, causing transient but much higher concentrations at discharge points (19). The results of groundwater contamination in karst aquifers may last for years (56); therefore the effects of chronic nonpoint source pollution can be expected to accumulate and magnify over time.

Keys to protecting the northern segment of the Edwards Aquifer include maintenance of the integrity of the watersheds (i.e., prevent pollutants from entering the watercourses) and delineation and protection of the localized high-porosity zones on the uplands that might be amenable to recharge--sinkholes, caverns, and loci of abnormal fracture density (86). Natural water-cycling through soils and vegetation should be used to mediate adverse effects on water quality and stream runoff, and impervious cover should be minimized. Understanding and conforming to natural hydrological cycles are especially important in this area, as the Balcones Escarpment is the locus of the largest flood-producing storms in the conterminous United States (9,63,86). The Edwards Aquifer (Balcones Fault Zone-Austin Region) from Hays County northward to Bell County has the highest ground-water pollution potential of the 10 major Texas aquifers (75).

The Texas Groundwater Protection Committee, a consortium of 9 state agencies, defines groundwater contamination as "the detrimental alteration of the naturally-occurring physical, thermal, chemical, or biological quality of ground water reasonably suspected of having been caused by activities or entities under the jurisdiction of the agencies identified in this report" (74). Improperly-completed and abandoned water wells, which constitute about 150,000 of the 600,000 wells which exist statewide, are a primary problem. They provide direct access from the surface to groundwater tables and, to the extent that they intersect different water bearing zones, also provide for interaquifer transfer of contaminants. Septic tanks, of which over one million older versions exist statewide, discharge large volumes of effluent directly to water-bearing units and are considered another major problem. Industrial waste-water impoundments, including those used for the disposal of salt water associated with oil and gas production, have only been regulated since 1969; abandoned facilities are not regulated and any problems which arise are dealt with on a case-by-case basis as they occur. Underground storage tanks are considered to be one of the principal contributing sources of ground-water pollution, placing a significant loading on the state's aquifers, due to their regional distribution and the high number which are estimated to be leaking (potentially 38,500 of 154,000). Municipal sanitary landfills may contribute significantly to ground-water pollution because until recently they lacked proper liners, leachate collection systems, and/or ground-water monitoring wells. High nitrates and pesticide levels in groundwater exist, but the extent of the problem is unknown because of the lack of a statewide agricultural chemical groundwater strategy, compounded by the unregulated use of high nitrate-bearing materials and waste waters in land-spreading operations (75).

Using the major categories of impacts affecting ground-water quality identified above, the following information has been compiled:

(1) **Land disposal of waste materials** - Bell County has 8 landfills and 1 processing facility, Travis County has 14 landfills and 5 processing facilities, and Williamson County has 8 landfills (L. Rodriguez, pers. comm.). There are no operating or permitted hazardous waste disposal facilities in the 3-county area.

(2) **Water wells** - water wells are considered one of the greatest sources of pollution of ground water in Texas (75). Wells have been drilled in Texas since settlement times, but driller's logs were not required to be filed prior to 1964 (G. Adair, pers. comm.). Since then, there have been 1,028 wells drilled in Bell County, 9,326 wells drilled in Travis County, and 3,523 wells drilled in Williamson County (G. Adair, pers. comm.). There are an additional 259 locater wells in Bell County, 1,263 locater wells in Travis County, and 646 such wells in Williamson County.

(3) **Sewage and waste water disposal systems and municipal collection lines** - There were 14 existing and 10 pending municipal sewage treatment facilities on the northern Edwards Aquifer in Travis and Williamson counties in 1985 (46).

Bell, Travis, and Williamson counties each maintain their own individual databases on septic systems and file periodic reports with the state (S. Hart, pers. comm.). The total number of septic systems in Bell County is not known (W. Farrell, pers. comm.,R. Bentley, pers. comm.). Data from the U.S. Census Bureau for 1990 indicate there were 14,166 households in the county hooked up to septic systems. There were 70 complaints of septic system malfunction filed between October, 1992 and August, 1994 (W. Farrell, pers. comm.). On 23 November 1994 a sign, posted by the Bell County Health Department, stood next to Big Boiling Springs in Salado, one of the two known localities for the Salado Springs Salamander, warning people not to engage in water recreation because of water quality and contamination concerns (90). This degradation appears to be a recent phenomenon (54, A.H. Price, pers. comm.).

Data from the U.S. Census Bureau for 1990 indicate there were 29,289 households in Travis County hooked up to septic systems. An inquiry regarding complaints, failure rates, and historical monitoring efforts was made to the Travis County Health Department in October, 1994; no answer has been received to date.

There were an estimated 20,000 septic systems in Williamson County in 1992, with perhaps 2,000 added since (P. Pinto, pers. comm.). The county did not begin licensing septic systems until about 15 years ago, and approximately 12,000 have been licensed since (P. Pinto, pers. comm.). Data from the U.S. Census Bureau for 1990 indicate there were 13,431 households hooked up to septic systems. There have been 90 complaints of system failures since March, 1991 (P. Pinto, pers. comm.). The highest density of septic systems (80-90% of the total) occur in the southwestern portion of the county, in the vicinity of the junction of Ranch Road 620 and U.S. Highway 183. These systems have been in place for more than 20 years and have the highest propensity countywide to fail (P. Pinto, pers. comm.).

(4) **Leaks and spills** - Spills of as little as 1,000 barrels have a reasonable chance of contaminating groundwater, and in karst aquifers such as this, one a single barrel of oil can poison over two square miles of the water table (56). It has been estimated that spills of this magnitude occur from existing oil pipelines over the Edwards Aquifer about every 15 months

(56).

There were about 200 confirmed leaking Underground Storage Tanks (UST's) in the 3-county area as of July 12, 1988 (75). According to the bi-weekly report of the Texas Natural Resource Conservation Commission for 28 September 1994, there were 526 leaking UST's in Travis County, 95 in Williamson County and 147 in Bell County.

During the 1987 fiscal year, there were 1592 known chemical spills statewide, 48 in Travis County and 10 in Williamson County (75). One of those in Travis County was one of only 5 statewide known or suspected to have impacted an aquifer. There were 77 reported chemical spills investigated by the Texas Natural Resource Conservation Commission and its predecessors during the years 1970-1985 in Travis County. There were 592 such spills from 1986 through the end of August, 1994; 122 were of unknown origin, and the majority of these were hydrocarbons of some sort (diesel fuel, gasoline, waste motor oil). There were 70 reported chemical spills investigated by the Texas Natural Resource Conservation Commission and its predecessors from 1982 through April, 1994 in Williamson County (but only six of these occurred prior to 1987), and 78 (26 prior to 1987) such spills through August, 1994 in Bell County. The Texas Water Commission said "...historically, the number of new ground-water contamination cases documented is far greater each year than the number of cases in which action has been completed to date. This trend is expected to continue for some time into the future. This is testimony that once ground water has been contaminated, it requires many years, and substantial cost, to remediate..." (74).

(5) Oil, gas, and mining activities - most such activities occur east of the Balcones Fault in the 3-county area and are believed to be minimally germane to the subject discussed herein. A notable exception are surface limestone mining operations, which are a potentially serious threat through either interference and/or interruption of normal springflows or actual physical destruction of the springs themselves. One historical locality near Round Rock is believed to have been destroyed by such activity (71) and another along the Middle Fork of the San Gabriel River was discovered during the course of a review of water permits for mining operations (12). Although additional springs inhabited by salamanders have subsequently been discovered in the area (L. Rodriguez, pers. comm.), it is possible that without appropriate precautionary measures mining operations here or elsewhere may impact these or previously unrecognized springs inhabited by salamanders.

(6) Agricultural practices - the Edwards Aquifer is considered the most susceptible aquifer to ground-water pollution from storm water runoff (75). In 1987, 21-40% of analyses in each of the 3 counties exceeded EPA national drinking water standards for nitrates. Relatively high nitrate concentrations in the western portion of the northern segment are "...generally an indication of surface pollution as a result of agricultural activity or local sources from sewage..." (33). A request was made to the Texas Department of Agriculture on 14 December 1994 for information pertaining to investigations of pesticide contamination in the 3-county area. The information exists, but is difficult to extract from their database; a resolution of this issue is pending.

(7) Ground-water withdrawals - this portion of the Edwards Aquifer is considered a "critical area", which means that it "is experiencing or is expected to have ground-water problems resulting from ground-water overdrafts" (38,75). Portions of western Williamson

County experienced ground-water level declines of 50-100 feet in artesian areas between 1975-1985, and portions of western Bell County experienced declines of greater than 100 feet during the same period. Travis County had 153 active ground water public-supply systems in February, 1991 (38); 5 of these, all municipalities, accounted for 26% of the volume used. Williamson County had between 21 and 50, and Bell County between 11 and 20 active ground water public-supply systems a decade ago (75). Many wells along the I-35 corridor show consistently low water levels after 1977 due to increased pumping for public supply and industrial purposes, "...ground-water recharge...is still essentially in balance with discharge from the aquifer. Ground-water pumping, however, is expected to increase because of the extremely rapid growth in population and attendant economic activity in parts of the region....current water-level trends are not expected to continue into the future" (3). Local ground-water shortages are expected to occur in Travis County prior to the year 2030 (38). Many municipalities are increasingly relying on surface or conjunctive sources of water; the latter category implies reliance on groundwater sources during times of drought.

The population of Williamson County was stable for the first 7 decades of this century,; then it more than doubled between 1970 and 1980. Total reported municipal and industrial pumpage from the aquifer increased gradually from 1 million gals./day in 1955 to 3.5 million gals./day in 1975; by 1984 it had increased to 12 million gals./day (23). Individual domestic wells, stock and irrigation wells, and some industrial and commercial wells were not included in these figures; if Texas Department of Water Resources estimates are added, the 1984 figure increases to 14.7 million gals./day. The major cause of increased pumpage after 1975 is watering of lawns and landscapes accompanying rapid suburban and urban growth in the county "...springflows have reached historical base levels, with some completely ceasing to flow, water levels in wells have fallen below record lows, and reports of water shortages and wells drying up have become more [and] more numerous during periods of below average precipitation lasting only 1 to 2 years" (23).

(8) Other factors - the effects of other possible ground-water pollution sources, such as open dumps, material stockpiles, containers, automobile junk yards, and residential disposals, are unknown.

The 1996 Draft Consensus Water Plan projects substantial growth in the human population and concomitant municipal water use in Travis, Williamson and Bell counties by the year 2050 (92; Table 1). Also, projections made by the City of Austin ca. 1980 indicate additional 250,000 people living within the area underlain by the aquifer by the year 2000 (62).

Paralleling the general trends outlined above, potential or realized threats to known salamander springs or springruns of the northern segment of the Edwards Aquifer have been cumulative and insidious. Generally excellent water quality in the main stem of Bull Creek was reported during the late 1970's (50). No point source discharges were identified but nonpoint source pollution problems existed, mainly in the downstream reaches along Loop 360. Data taken on lower Bull Creek and Walnut Creek between 1975 and 1985 revealed concentrations of physical organics and inorganics, nutrients, indicator bacteria, inorganic trace elements, synthetic organic compounds, and radiochemical constituents to be larger during storm flow than during base flow (79). In addition, the base flow median concentrations for each were significantly smaller than those during storm flow. Inorganic trace elements and synthetic organic compounds are of concern because of their toxic effects on aquatic life, and their persistence and bioconcentration in the environment. All eighteen of the trace elements and 22 of the 42 synthetic organic compounds tested for were detected, and they occurred more frequently and at higher concentrations at sample sites draining more urban basins. Higher concentrations of arsenic, barium, iron, manganese, chlordane, DDD, DDE, DDT, dieldrin, diazinon, malathion, 2,4-D, 2,4,5-T, atrazine, and prometone were significantly correlated with increasingly urbanized watersheds. The concentrations and densities of suspended solids, biochemical oxygen demand, total organic carbon, total nitrogen, total phosphorus, fecal coliforms, and fecal streptococci increased with increased impervious cover under all flow regimes (79). Water quality has continued to decline in springs recharged from developed areas within the Bull Creek watershed (30).

The Bull Creek watershed had a greater proportion of impervious cover (11.5%) than the Barton Creek watershed (7%) (76). In 1979, approximately 8.5% of the Bull Creek watershed was developed; by 1984 that figure was 22%, and approximately 30-50% of that was impervious cover. Successive editions of the USGS 7.5' Jollyville quadrangle map reveal that approximately 30% of that area has been urbanized between 1973 and 1987. Development has already surrounded much of the eastern headwater tributary of Bull Creek. Plans are underway to urbanize the remainder of the headwater watersheds of Bull Creek; originally included in those plans have been wastewater lines and retention ponds in the stream channels themselves (D. Johns, pers. comm., W.J. Quinn, pers. comm., L. O'Donnell, pers. comm.). Several springs shown on early editions of the Jollyville and nearby quad maps have been physically destroyed or impounded (6,71, A.H. Price, pers. comm.). At least one cave known to have salamanders on the Jollyville Plateau has been filled in (71, W.R. Elliott, pers. comm., J.R. Reddell, pers. comm.).

One salamander spring in the Tanglewood Estates subdivision, considered highly impacted when first visited in 1991 (A.H. Price, pers. comm., P.T. Chippindale, pers. comm., D.M. Hillis, pers. comm.), had the highest specific conductance values measured in one study (67), indicative of high levels of unattenuated pollution. Jollyville Plateau Salamanders were commonly found in the springs and spring runs of Stillhouse Hollow, the prospective type-locality for the species and a City of Austin Nature Preserve, from 1987 until 1992 (A.H. Price, pers. comm., P.T. Chippindale, pers. comm., D.M. Hillis, pers. comm.). Beginning in 1993, the species became less common, which has coincided with repeated incidences of a foamy discharge from the main headwaters spring, first noticed on 19 November 1992 and continuing since after significant rainfall events (M. Sanders, pers. comm.). Water samples indicated contamination by petroleum hydrocarbons possibly related to a nearby underground petroleum storage tank (93) as well as other compounds typically found in storm water runoff from urbanized watersheds (D. Johns, pers. comm.). Salamanders had subsequently not been found in the main springs since June of 1993 nor in a smaller spring nearby since December of 1993 (M. Sanders, pers. comm.), but monthly surveys begun in March, 1995, have reconfirmed their presence (A.H. Price, pers. comm.,M. Sanders, pers. comm.). On March 20, 1994, a blockage in the gravity-flow sewage line in the adjacent drainage, Barrow Hollow, occurred upstream from where a dense concentration of Jollyville Plateau Salamanders was discovered in 1991. Raw sewage spilled out into the springrun for about 2 weeks because response crews couldn't get the necessary equipment to the site due to the rugged topography. The site has shown no evidence of viable aquatic macro-invertebrate or vertebrate populations since (A.H. Price, pers. comm., M. Sanders, pers. comm.).

Some threats to known spring and springrun locations of the Georgetown Salamander and Salado Springs Salamander were mentioned in the preceding discussion of factors affecting groundwater quality. Three historic salamander springs occur in San Gabriel Park along the banks of the San Gabriel River within the city of Georgetown in Williamson County (72). A juvenile specimen was collected there in 1992 (13,14). These springs are heavily impacted and do not support a viable population of the Georgetown Salamander. This is perhaps the result of drilling of a nearby well by the City of Georgetown and the impoundment of the San Gabriel River just downstream. Springflow has been markedly reduced and the river raised to the point that the direction of flow is often reversed, with river water flowing into the springs (6,33, A.H. Price, pers. comm., P.T. Chippindale, pers. comm., D.M. Hillis, pers. comm.).

The Cowan Creek Spring locality for the Georgetown Salamander was discovered because the tract on which it occurs is being considered for a large-scale residential and commercial development (A.H. Price, pers. comm., 94).

At least 4 potential groundwater contamination incidents in the vicinity of Salado Springs in Bell County have been investigated by the Texas Natural Resource Conservation Commission and its antecedents since 1989 (R. Wahl, pers. comm.). Sometime prior to 1 November 1991 a 550 gal. petroleum UST was removed from the grounds of the Stagecoach Inn, located at 1 Main Street in Salado (95), at which time it was discovered to have been leaking. Total petroleum hydrocarbons measured from tank bottom and backfill on that date were 9.0 and 5.0 mg/kg, respectively. The Stagecoach Inn sits on the bank of Salado Creek literally across the street from Big Boiling Springs. There is another surface springrun coursing through the property, and a subterranean wellhouse access shaft nearby goes down about 50 feet to the water table (A.H. Price, pers. comm., P.T. Chippindale, pers. comm.).

Another spill of approximately 700 gal. of unleaded gasoline occurred at Smith Texaco, located at I-35 and Thomas Arnold Drive on 16 January 1989 (96). This site is directly across Salado Creek from Robertson Springs and about 1/4 mi. upstream from Big Boiling Springs. The effects of the spill on groundwater were unknown; the spill drained into Salado Creek and "...was controlled with no ill affects [sic] on the stream" (97). On 3 July 1991 about 400 gal. of unleaded plus gasoline spilled from this site, now abandoned by its owner, Big Chief Distributing Company (98). This time groundwater was impacted although the extent is unknown; 2 nearby domestic wells were contaminated and high levels of BTEX and TPH were found all over the site. On 20 July 1991, a contractor hired by TNRCC removed and destroyed 5 petroleum USTs. Correspondence in the case file dated July and August, 1993 states that the owner is claiming financial inability to conduct corrective action and is applying for state-lead (= equivalent to Federal Superfund) status for the site, and that TNRCC is running out of funds to perform such action. On 31 December 1993 Big Chief Distributing Company was referred to the Enforcement Section of TNRCC for failure to take corrective action; the case is still pending.

The most prominent threat to amphibian populations worldwide is habitat destruction, followed by environmental contaminants (80). In addition to outright physical destruction of the spring habitats of *Eurycea* discussed above, "improvements" to springs are likely to be detrimental; predatory fish have a significant negative effect on larval salamander populations (29,31,52,61,69,73). Although specific data for species of *Eurycea* are lacking, significant effects on amphibians in general are known from a wide variety of pollutants, including those discussed in this paper (18,20,26,40,51,53,70,99). Water quality factors likely to affect the species of *Eurycea* discussed in this report have been summarized (51). Sublethal effects on other aquatic organisms and incremental steps towards dysfunction and collapse of these sensitive spring ecosystems are of at least equal concern (8,28,36,41,47,64,65,81,84,85,100). A wide variety of specific heavy metals, pesticides, petroleum hydrocarbons, and other anthropogenic compounds are known to have acute toxic effects on many aquatic organisms, including some on which these *Eurycea* depend for food or other ecosystem services (4,32,59,60,66,78,83,88). Urbanization dramatically synergizes the effects of these factors on aquatic ecosystems (42,82). For example, *Eurycea sosorum,* and the amphipods which are a staple food item, recolonized the bottom of Barton Springs pool following the chlorine spill of September, 1992. Amphipods were particularly abundant on the vascular plants which had been established as part of the City of Austin's attempt to revegetate the area. These plants were mistakenly removed a year later by members of the University of Texas Dive Club engaged in volunteer pool maintenance activities, and both amphipods and salamanders subsequently declined precipitously in abundance. The plants have recolonized the area, but the amphipods and salamanders have not recovered. Significantly-elevated levels of sediment emerging from the Parthenia outlets of Barton Springs have covered the bottom of the pool in the interim, associated with the increased rate of sedimentation occurring within the segment of the aquifer feeding Barton Springs (24). Preliminary results of tests conducted for the city indicate that these sediments contain elevated levels of polyaromatic hydrocarbons (PAH) which are toxic to amphipods (R. Hansen, pers. comm., J. Dwyer, pers. comm.). Elevated levels of these compounds are characteristic of roadways and urban areas.

Ecological Projections

Criticism has been leveled at the work done on central Texas *Eurycea* for not providing explicit threats assessments in the form of "x population of salamanders is subject to y threats under a z time frame" (101), a task fraught with difficulty and worth several lifetimes of work (102). Nevertheless, the central Texas hemidactyliine salamanders which are the subjects of this report are a "focal taxon" (17,34,35,37,49,58,81) for aquatic ecosystems throughout the Edwards Plateau region. This means that the salamanders can serve as sensitive bioindicators for conservation planning efforts of the health of their habitats and indirectly of the health of the other species that are a part of these aquatic ecosystems. Detailed earlier in this report are some of the factors which are likely to affect the persistence of populations of the distinctive group of *Eurycea* north of the Colorado River. These populations are more seriously and imminently threatened with extinction than most of the remaining populations of *Eurycea* south of the Colorado River (10-15, A.H. Price, pers. comm., P.T. Chippindale, pers. comm., D.M. Hillis, pers. comm.). It is plainly evident that the existing or possible information pertinent to the subjects of this report has not been exhausted (102), and it is always nice to have more data (45,48,51,57,99); nevertheless the implications of the evidence which has been gathered to date are clear. The need to have these species included in ongoing conservation planning efforts is not new (10-15, A.H. Price, pers. comm., P.T. Chippindale, pers. comm., D.M. Hillis, pers.

comm.,103). Based on the evidence presented in this report and without evaluating the degree of protection associated with recently established regulations by TNRCC or additional conservation planning, the following projections concerning the future of these salamanders can be made:

1. Populations of the **Jollyville Plateau Salamander** inhabiting the Bull Creek drainage will become extinct within the first decade of the next century as the result of declining water quality, physical destruction of habitat, and the complete urbanization of the watershed. The small, isolated populations located along Walnut Creek and Brushy Creek will follow by the year 2010 from similar causes or random stochastic events.

2. As urbanization and its attendant effects on habitat quality proceed northward in Travis County, populations of the **Jollyville Plateau Salamander** inhabiting most of the Cypress Creek watershed will become extinct by the year 2020. The populations of the **Jollyville Plateau Salamander** inhabiting the headwater springs of the northern tributary of Cypress Creek, located within the Travis Audubon Society Wildlife Sanctuary, and the springs and springruns in Long Hollow Creek, located within the Ruth P. Lehmann Preserve (Texas Nature Conservancy) and the Wheless Tract (Lower Colorado River Authority) will probably survive, so long as these areas remain wilderness preserves. However, these populations can be expected to disappear as well should the watersheds supplying them follow the above scenario, or in the event of an extended drought.

3. Salamanders within the Buttercup Creek cave system will become increasingly difficult to find as urbanization and development in the Cedar Park area increases pollutant loading while decreasing water and nutrient input to the subterranean ecosystem. This population may well become extinct before its status can be determined.

4. Populations of the **Georgetown Salamander** will remain viable as an inverse function of the amount, rate, and nature of development surrounding the springs they inhabit. As mentioned above, the population inhabiting the springs within the City of Georgetown is gone from a functional standpoint, and the future of the population inhabiting Cowan Creek Spring depends upon the timetable of the development surrounding it. The populations inhabiting the springs along the south shore of Lake Georgetown should remain viable for the long-term; however, should unrestricted commercial and/or resort development occur in this limited area, then these populations will also be in jeopardy of surviving the first half of the 21st century.

5. The future of the **Salado Springs Salamander** is the most difficult to predict. Specimens have been increasingly difficult to find since 1991, but prior to the work cited herein only one specimen was known to science. Because of its extremely circumscribed range and subterranean habits, combined with the factors outlined

herein, this species may slip out of existence before anyone notices.

Conclusions and Management Recommendations

Even if these projections (above) overestimate the threats by an order of magnitude, the potential for these species, springs, and ecosystems to cease remain so serious as to warrant a great deal of public attention, proactive conservation planning and monitoring. Management recommendations based on the information in this final report are discussed elsewhere (102); Items 3. – 7. below are addressed in detail in that report.

1. *Eurycea* populations north of the Colorado River are highly distinct from those to the south. The northern population group should be treated as a completely difference species group from other *Eurycea* species for conservation purposes. Please refer to Part II of this report for additional detail.
2. Keys to protecting the northern segment of the Edwards Aquifer include (a) maintenance of the integrity of the watershed and (b) delineation and protection of the localized high-porosity zones on the uplands that might be amenable to recharge, i.e., sinkholes, caverns, and loci of high-fracture density.
3. An ecosystem approach is required for entire watersheds in order to conserve *Eurycea* species. An ecosystem management plan along with target research goals needs to be developed for the entire Edwards Aquifer system.
4. Monitoring and management plans for water quality and water quantity need to be developed and implemented as soon as possible.
5. Additional life history studies are needed, most urgently, for the Barton Springs Salamander and the Jollyville Plateau Salamander.
6. Dye tracing and other studies relating to surface water/groundwater interactions are required to identify and delineate surface sources of waters from springs. Knowledge of flow dynamics during normal precipitation and storms is essential for long-term management.
7. Once a base-line is established from items (4) and (6), regulatory management authorities, for example, an aquifer district, should be given the responsibility of writing and implementing guidelines to mitigate development impacts associated with water quality, impervious cover and watershed dynamics.

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Appendix I.

Notes given below are based on a review of this report by Texas Natural Resources Conservation Commission staff, dated 5 October 1999.

1. A more recent map of the Edwards Aquifer is available from the Texas Water Development Board in their publication Aquifers of Texas. It is available on the web site http://www.twdb.state.tx.us.

2. More recent information on hydology and impacts to Salado Creek is available in P.S. Rodusky 1997, Non-point source pollution and implications regarding groundwater-surface water interactions in Salado Creek Basin, Texas. MS thesis, Baylor University.

3. The Texas Natural Resources Conservation Commission's 30 TAC 285 rules, which regulate On-Site Sewage Facilities, were revised in February, 1997. The authorized agents in the affected counties can provide information on their programs which must be as stringent as the Commission's 30 TAC 285 rules.

4. The "critical area" designation in the section on groundwater withdrawals is no longer used and has been replaced with "priority groundwater management area". The report states that this area is considered to be a "critical area". In fact, this area was determined to not be a critical area by the Texas Water Commission on 17 October 1990; however, continued monitoring and assessment of the area is ongoing. As required under Senate Bill 1 from the 75th legislative session, the area is being reevaluated. The Texas Water Development Board completed its report titled Updated Evaluation of Water Resources in Bell, Travis, Williamson, and parts of Bastrop, Lee, and Milam Counties, Texas (Open-File Report 99-01) in July 1999 and the Texas Parks and Wildlife Department completed its report titled Evaluation of Selected Natural Resources within Williamson and Parts of Adjacent Counties, Texas in January 1999. Both reports contain updated information regarding groundwater withdrawals including projected demands and population projects. Groundwater is part of the total water resource for the area, with surface water being a major part of the existing and projected supply to meet demands of the area. The use of both surface and groundwater needs to be analyzed to determine the full potential threat to the species.

5. The Texas Natural Resources Conservation Commission participated in a cooperative study of Bull Creek with the City of Austin this summer (1999). Results of that effort, when completed, will help answer questions about the current condition of Bull Creek.

6. Rules and regulations adopted and enforced by the Texas Natural Resources Conservation Commission which cover the Edwards Aquifer are available at the agency web site http://www.tnrcc.state.tx.us/EAPP/index.html .

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FIGURE AND TABLE LEGENDS

Figure 1. Location of the Edwards Aquifer in central Texas, after (33).

Figure 2. Distribution of perennibranchiate hemidactyliine plethodontid salamanders of the genus *Eurycea* in central Texas. Dots indicate the location of selected epigean populations and squares the location of selected troglobytic populations.

Figure 3. Locations of the Jollyville Plateau, Georgetown, and Salado Springs salamanders in Travis, Williamson, and Bell counties, Texas.

Figure 4. Map of the extent of the Edwards Aquifer in Bell, Travis, and Williamson counties, Texas; after (3).

Figure 5. Map of the Bull Creek watershed in central Travis County, Texas; after (30).

Table 1. Projections of human population growth and concomitant municipal water use in Bell, Travis, and Williamson counties, Texas, from the 1996 Draft Consensus Water Plan; after (105).

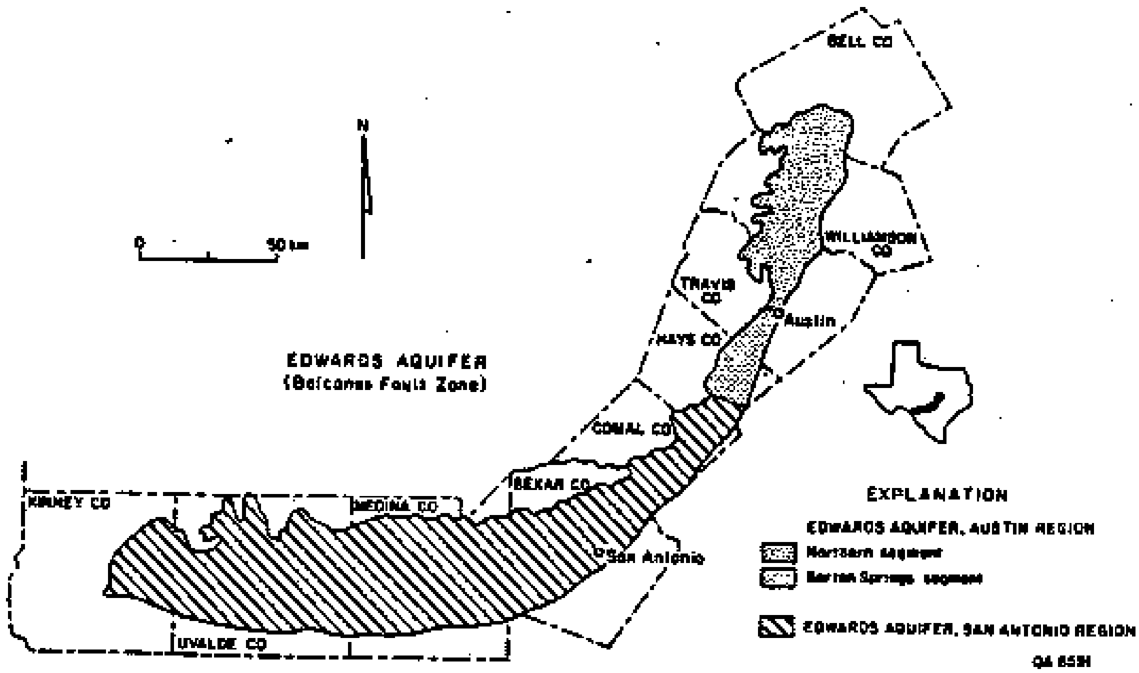


Figure 1. Division of the Edwards aquifer according to the Texas Department of Water Resources (1978).

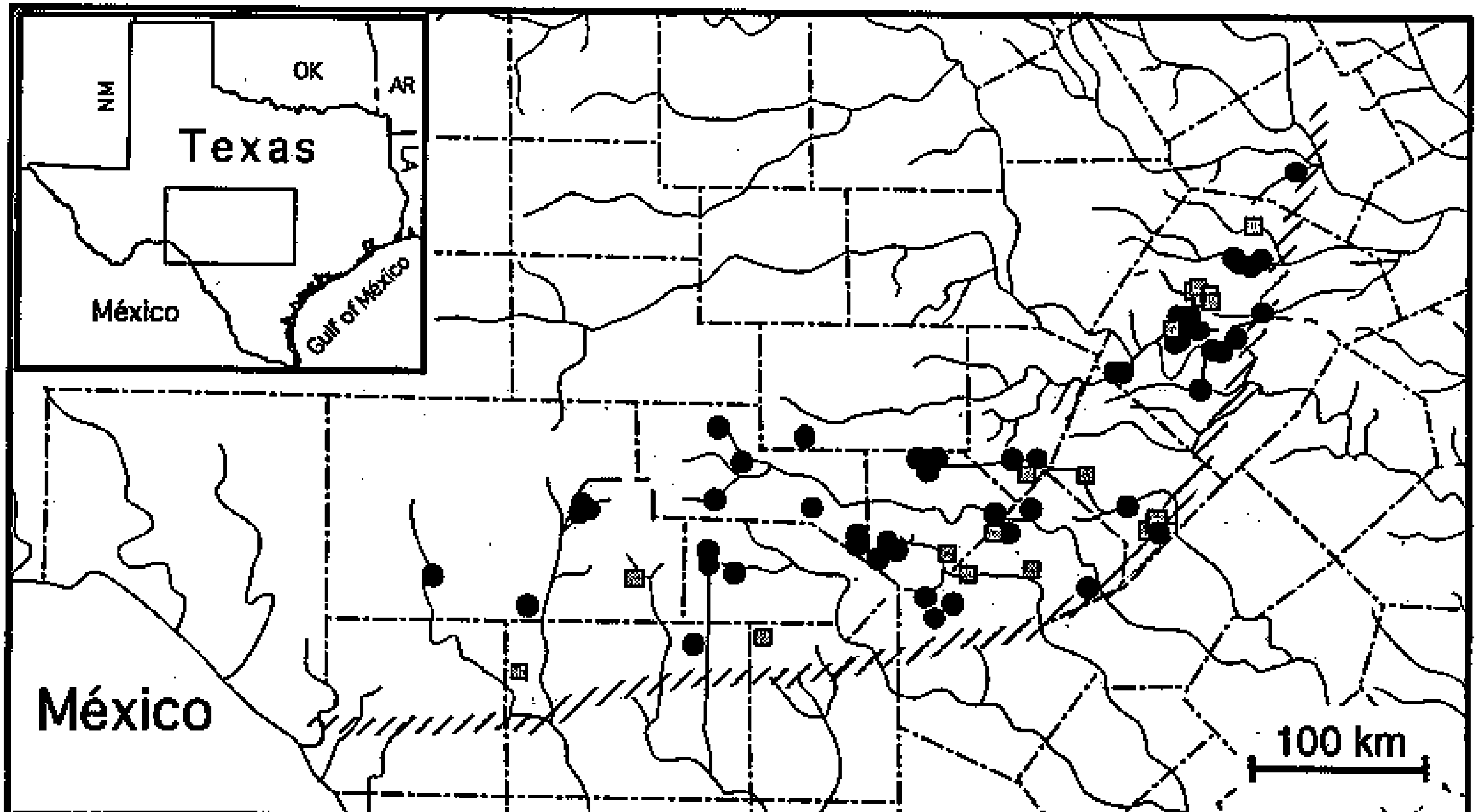


Figure 2

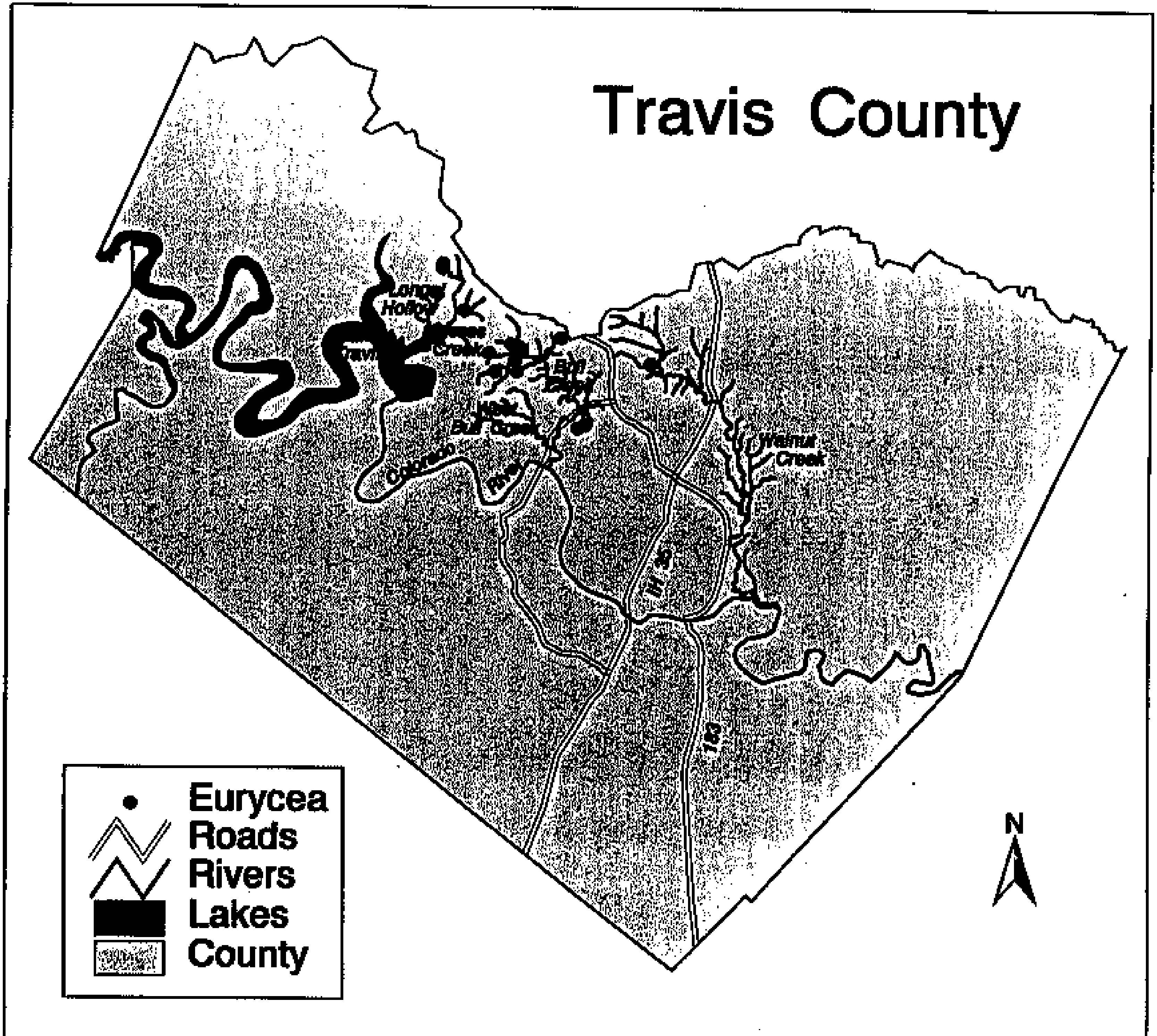

Travis County
Walnut Creek
IH 35
183
N
Eurycea
Roads
Rivers
Lakes
County

Figure 3a

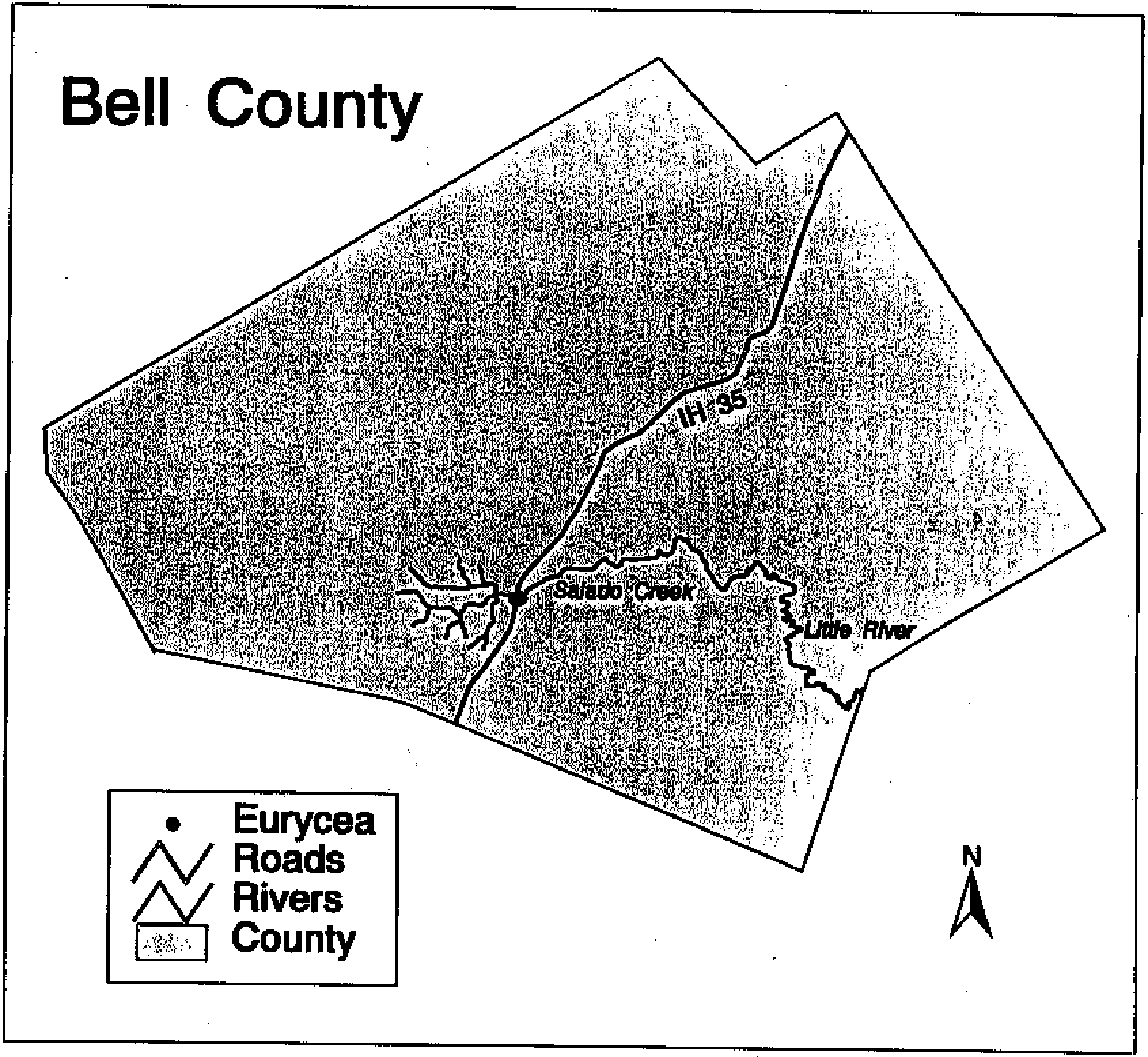

Bell County
IH 35
Salado Creek
Little River
Eurycea
Roads
Rivers
County
N

Figure 3b

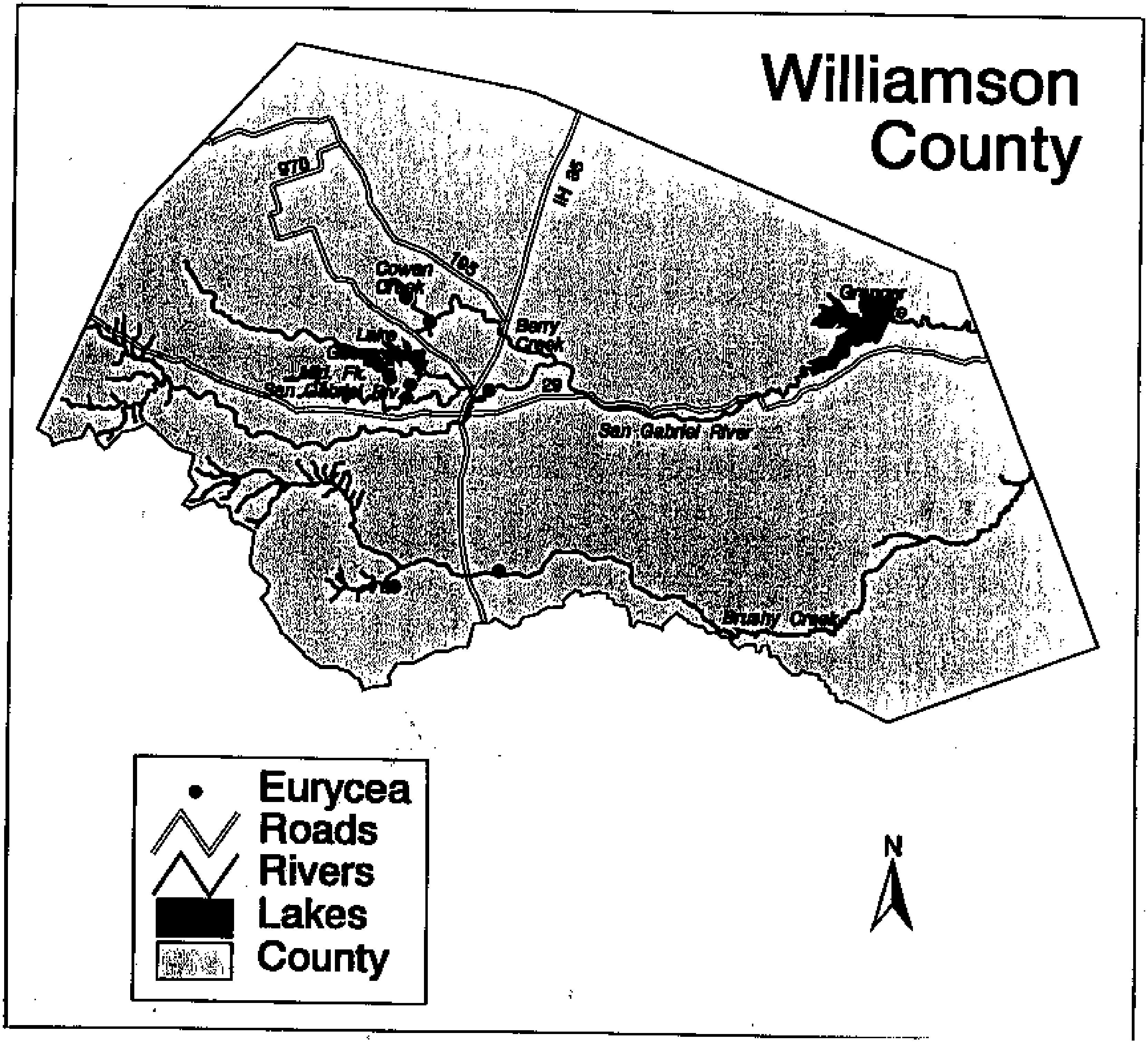
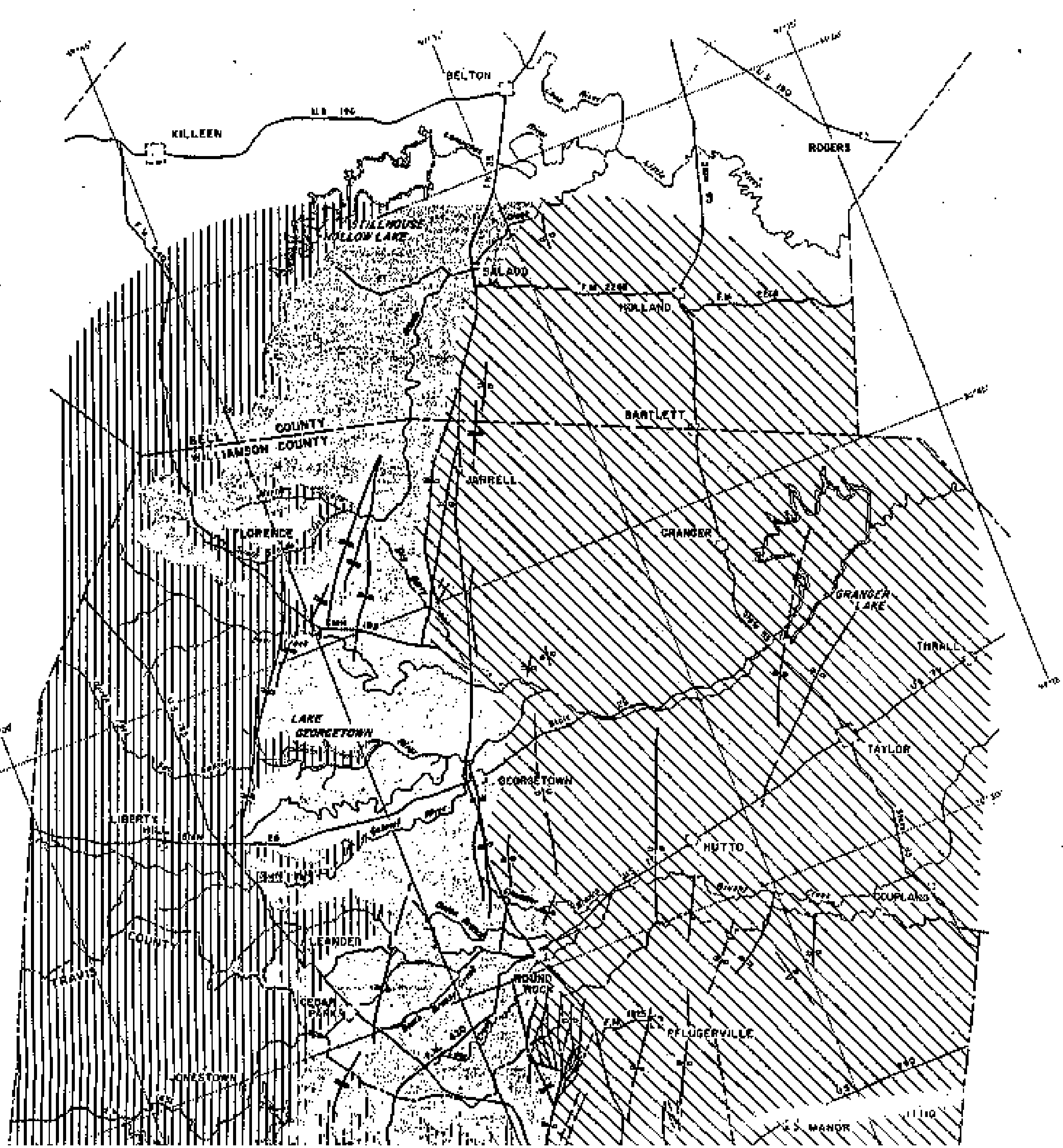
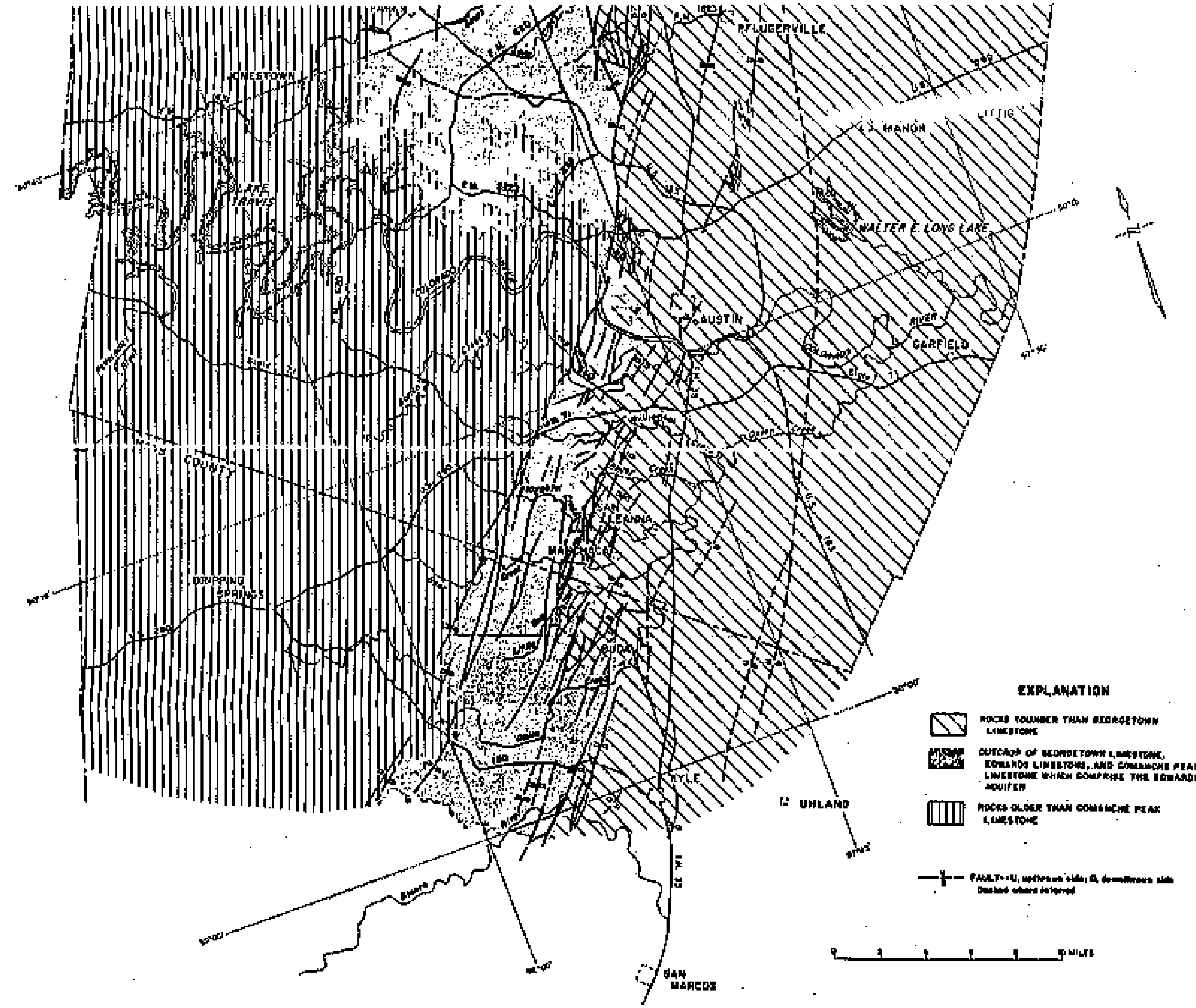

Williamson County
970
195
IH 35
Cowan Creek
Berry Creek
29
San Gabriel River
Brushy Creek
Eurycea
Roads
Rivers
Lakes
County
N

Figure 3c

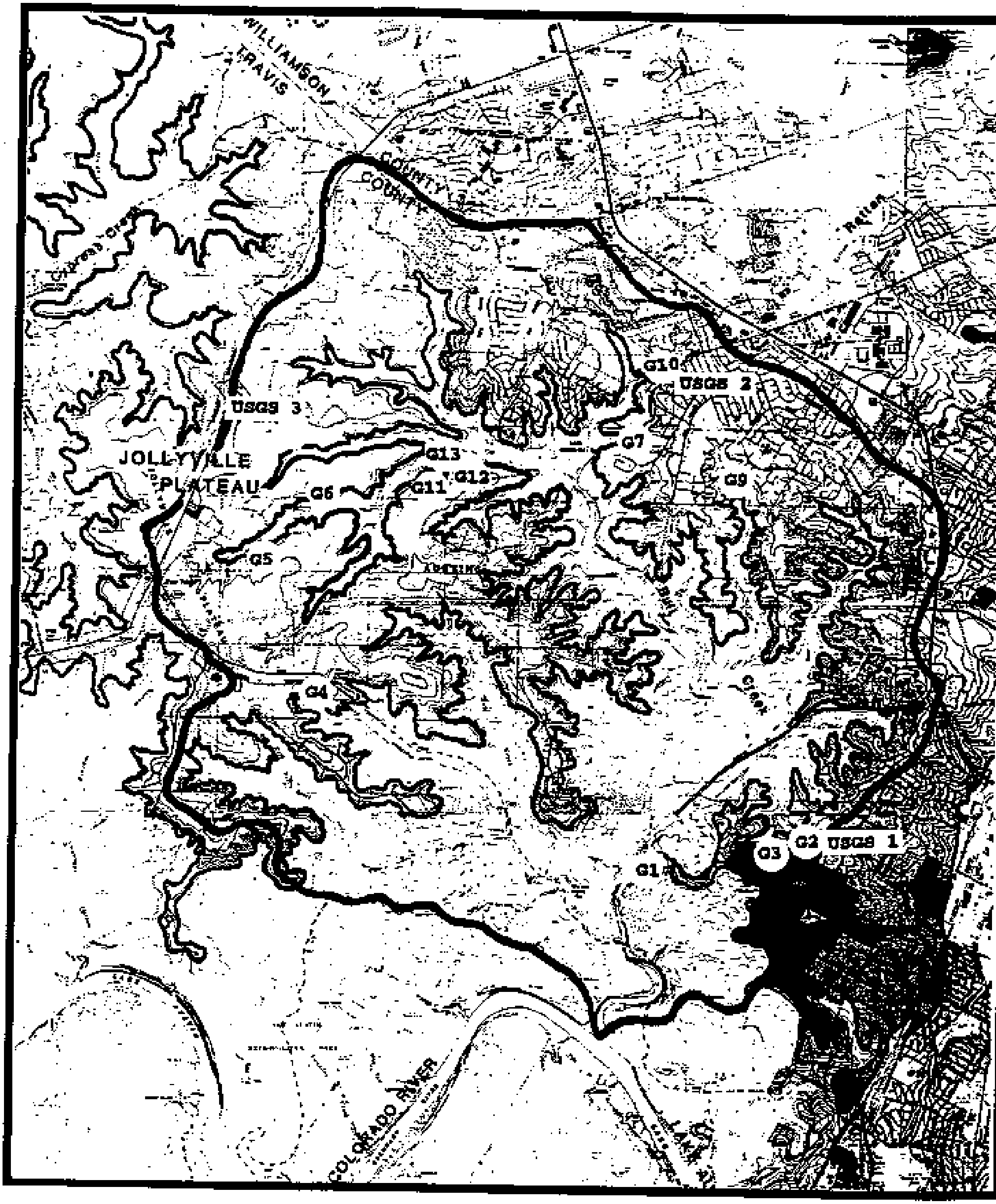


BELTON
KILLEEN
ROGERS
STILLHOUSE HOLLOW LAKE
SALADO
HOLLAND
BARTLETT
BELL COUNTY
WILLIAMSON COUNTY
JARRELL
FLORENCE
GRANGER
GRANGER LAKE
THRALL
LAKE GEORGETOWN
GEORGETOWN
TAYLOR
LIBERTY HILL
HUTTO
COUPLAND
LEANDER
ROUND ROCK
CEDAR PARK
PFLUGERVILLE
TRAVIS COUNTY
MANOR



Hydrogeologic Map

Figure 4



LEGEND

— — Creeks and Tributaries

G1 City of Austin Sampling Site

USGS 1 USGS Sampling Site

SCALE

0 1 2 MILES

N

BULL CREEK SPRING SAMPLING SITES

Base Map: "Recharge Zone of the Northern Edwards Aquifer Near Austin, Texas" by Garza and Stagle, City of Austin, 1988.

1996 CONSENSUS TEXAS WATER PLAN
PROJECTIONS OF POPULATION AND MUNICIPAL WATER USE
WATER USE UNITS: ACRE-FEET
*** DRAFT -- SUBJECT TO REVISION ***

Table 1

DRAFT

COUNTY: 014 BELL

SERIES	HISTORICAL 1980	HISTORICAL 1990	PROJECTED 2000	2010	2020	2030	2040	2050
Population	157889	191088						
Water Use	27241	33163						
*** MIGRATION RATE .0**								
Population..............			214404	233072	253310	264877	276371	285037
Avg. Weather Condition			37450	39876	42628	44921	46325	47790
Below Normal Rainfall			42919	45428	48715	51619	53051	54730
Average/Conservation			35541	35825	36135	37008	37355	38134
Below Normal/Conserv.			40876	41165	41624	42859	43309	44467
Average/Advanced Cons			34377	33290	32907	34264	34916	35582
Below Normal/Advanced			39504	38338	37882	39426	40311	41115
(With Plumbing Code Only)								
Average/Conservation			36299	37264	38540	39652	40039	40949
Below Normal/Conserv.			41768	43015	44649	46153	46763	47893
Average/Advanced Cons			35476	36140	37525	39128	39811	40630
Below Normal/Advanced			41142	41891	43612	45631	46536	47572
*** MIGRATION RATE .5**								
Population..............			213307	234147	256764	273376	283336	298704
Avg. Weather Condition			37247	40062	43215	46023	47500	50096
Below Normal Rainfall			42683	45840	49387	52684	54401	57378
Average/Conservation			35349	35987	36613	37904	38315	40006
Below Normal/Conserv.			40656	41339	42265	43927	44421	46629
Average/Advanced Cons			34207	33474	33379	35154	35813	37297
Below Normal/Advanced			39290	38516	38449	40442	41349	43136
(With Plumbing Code Only)								
Average/Conservation			36103	37440	39112	40639	41071	42924
Below Normal/Conserv.			41537	43219	45281	47302	47969	50204
Average/Advanced Cons			35482	36309	38060	40011	40833	42570
Below Normal/Advanced			40920	42089	44232	46673	47735	49852
*** MIGRATION RATE 1.0**								
Population..............			211605	234766	256756	271633	281161	295069
Avg. Weather Condition			36943	40169	43214	45726	47135	49483
Below Normal Rainfall			42340	45961	49385	52346	53981	56673
Average/Conservation			35165	35978	36626	37680	38020	39517
Below Normal/Conserv.			40296	41449	42236	43661	44097	46044
Average/Advanced Cons			33936	33563	33395	34945	35557	36841
Below Normal/Advanced			38972	38540	38448	40200	41048	42610
(With Plumbing Code Only)								
Average/Conservation			35864	37538	39108	40377	40768	42398
Below Normal/Conserv.			41260	43331	45276	46996	47614	49587
Average/Advanced Cons			35174	36348	38059	39754	40518	42067
Below Normal/Advanced			40568	42139	44229	46374	47364	49259
*** MOST LIKELY SERIES**								
Population..............			213307	234147	256764	273376	283336	298704
Avg. Weather Condition			37247	40062	43215	46023	47500	50096
Below Normal Rainfall			42683	45840	49387	52684	54401	57378
Average/Conservation			35349	35987	36632	37923	38315	40006
Below Normal/Conserv.			40636	41339	42245	43946	44441	46648
Average/Advanced Cons			34207	33474	33398	35173	35832	37297
Below Normal/Advanced			39290	38516	38468	40461	41368	43136
(With Plumbing Code Only)								
Average/Conservation			36103	37440	39112	40639	41090	42926
Below Normal/Conserv.			41537	43219	45281	47302	47988	50204
Average/Advanced Cons			35462	36309	38060	40011	40833	42589
Below Normal/Advanced			40900	42089	44232	46673	47735	49871

1996 CONSENSUS TEXAS WATER PLAN
PROJECTIONS OF POPULATION AND MUNICIPAL WATER USE
WATER USE UNITS: ACRE-FEET
***** DRAFT -- SUBJECT TO REVISION *****

COUNTY: 227 TRAVIS

DRAFT

SERIES	HISTORICAL 1980	HISTORICAL 1990	PROJECTED 2000	PROJECTED 2010	PROJECTED 2020	PROJECTED 2030	PROJECTED 2040	PROJECTED 2050
Population	419573	378407						
Water Use	87800	114970						
* MIGRATION RATE .0								
Population..............			643972	695030	745378	783404	804557	810180
Avg. Weather Condition			130283	140584	150743	158422	162693	163823
Below Normal Rainfall			159375	171943	184344	193710	198922	200305
Average/Conservation			125409	129844	133862	137835	138762	138816
Below Normal/Conserv.			153985	159568	164786	169687	171456	171746
Average/Advanced Cons			122364	122218	124444	129032	130843	130740
Below Normal/Advanced			149328	150308	152200	158193	161424	161434
(With Plumbing Code Only)								
Average/Conservation			127959	134170	140537	144946	146077	146181
Below Normal/Conserv.			157052	165527	174136	180237	182306	182563
Average/Advanced Cons			125803	130953	137118	142375	145174	145168
Below Normal/Advanced			154894	162311	170718	177664	181404	181649
* MIGRATION RATE .5								
Population..............			647072	712776	787914	854324	902997	953878
Avg. Weather Condition			130903	144142	159274	172650	182445	193055
Below Normal Rainfall			160135	176300	194791	211132	223108	236098
Average/Conservation			126209	133016	141301	149378	155705	163676
Below Normal/Conserv.			154720	163501	173377	184934	192270	202396
Average/Advanced Cons			122943	125301	130724	140597	146806	156026
Below Normal/Advanced			150139	154103	160805	172364	181138	190475
(With Plumbing Code Only)								
Average/Conservation			128573	137560	148361	157035	162944	172243
Below Normal/Conserv.			157803	169720	183879	195518	203611	215288
Average/Advanced Cons			126402	133600	144863	155125	162903	171173
Below Normal/Advanced			155635	165758	180382	193609	203567	214216
* MIGRATION RATE 1.0								
Population..............			649722	727901	823636	917382	995413	1084190
Avg. Weather Condition			131435	147174	166438	185301	200989	218799
Below Normal Rainfall			160785	180014	203564	226626	245810	267623
Average/Conservation			126719	135810	146878	160307	170556	185460
Below Normal/Conserv.			155334	166259	181145	198452	211815	229385
Average/Advanced Cons			123442	127933	136593	150012	161829	174672
Below Normal/Advanced			150748	157364	168036	185013	199567	215877
(With Plumbing Code Only)								
Average/Conservation			129091	139774	154255	168329	179490	194175
Below Normal/Conserv.			158444	172616	191381	209853	224315	242996
Average/Advanced Cons			126911	136302	151372	166476	179575	193981
Below Normal/Advanced			156263	169163	186501	207801	224400	242801
* MOST LIKELY SERIES								
Population..............			744081	892064	1096330	1288442	1413421	1550521
Avg. Weather Condition			150340	180078	221126	259739	284859	312367
Below Normal Rainfall			183952	220326	270544	317776	348507	382189
Average/Conservation			144913	165280	193844	223369	241636	263453
Below Normal/Conserv.			177018	202579	239478	276907	300616	327706
Average/Advanced Cons			140473	155635	181527	210309	229058	249468
Below Normal/Advanced			172438	191815	222192	259494	281465	308348
(With Plumbing Code Only)								
Average/Conservation			146941	170010	203837	234919	254472	277355
Below Normal/Conserv.			180555	210257	253254	292958	318121	347176
Average/Advanced Cons			144445	166871	200011	233297	254272	277077
Below Normal/Advanced			178057	207118	249428	291337	317918	346896

Table 1

1996 CONSENSUS TEXAS WATER PLAN
PROJECTIONS OF POPULATION AND MUNICIPAL WATER USE
WATER USE UNITS: ACRE-FEET
*** DRAFT -- SUBJECT TO REVISION ***

DRAFT

COUNTY: 246 WILLIAMSON

SERIES	HISTORICAL 1980	HISTORICAL 1990	PROJECTED 2000	PROJECTED 2010	PROJECTED 2020	PROJECTED 2030	PROJECTED 2040	PROJECTED 2050
Population	76521	139551						
Water Use	13388	24482						
* MIGRATION RATE .0								
Population.............			153697	162694	169412	168703	161809	149089
Avg. Weather Condition			27042	28711	29929	29727	28505	26424
Below Normal Rainfall			30666	32596	34019	33721	32330	29969
Average/Conservation			25704	25781	25481	24764	23033	21270
Below Normal/Conserv.			29125	29467	29246	28307	26417	24484
Average/Advanced Cons			24793	23990	23329	22735	21544	19786
Below Normal/Advanced			28134	27357	26626	25967	24618	22642
(With Plumbing Code Only)								
Average/Conservation			26197	27005	27411	26636	24821	22912
Below Normal/Conserv.			29620	30889	31497	30630	28648	26456
Average/Advanced Cons			25775	26119	26712	26102	24797	22764
Below Normal/Advanced			29398	30004	30800	30098	28622	26288
* MIGRATION RATE .5								
Population.............			187153	240322	303079	367597	420983	469419
Avg. Weather Condition			32926	42410	53545	64774	74165	82697
Below Normal Rainfall			37341	48148	60856	73477	84115	93791
Average/Conservation			31018	37486	44823	52986	59733	66467
Below Normal/Conserv.			35288	42822	51294	60896	68807	76287
Average/Advanced Cons			29910	35080	41145	49232	55908	61882
Below Normal/Advanced			34075	40066	47032	56310	64023	70821
(With Plumbing Code Only)								
Average/Conservation			31722	39252	48189	57009	64326	71666
Below Normal/Conserv.			36134	44990	55502	65712	74272	82758
Average/Advanced Cons			31219	38329	47307	56560	64323	71138
Below Normal/Advanced			35633	44068	54616	65263	74270	82231
* MIGRATION RATE 1.0								
Population.............			226843	347410	520306	762479	1025502	1408249
Avg. Weather Condition			39912	61307	91922	134355	180662	248089
Below Normal Rainfall			45258	69603	104472	152408	204898	281372
Average/Conservation			37379	53441	75806	108995	145295	198981
Below Normal/Conserv.			42601	61156	87266	125063	166793	228542
Average/Advanced Cons			36037	50375	70408	101328	135467	185762
Below Normal/Advanced			41066	57657	80230	116051	154907	212579
(With Plumbing Code Only)								
Average/Conservation			38215	55996	81611	117663	156643	213587
Below Normal/Conserv.			43565	64294	94163	135518	180850	246870
Average/Advanced Cons			37611	54794	80767	116567	156640	213526
Below Normal/Advanced			42958	63090	93319	134626	180877	246809
* MOST LIKELY SERIES								
Population.............			187153	240322	303079	367597	420983	469419
Avg. Weather Condition			32926	42410	53545	64776	74165	82697
Below Normal Rainfall			37341	48148	60856	73477	84115	93791
Average/Conservation			31018	37476	44795	52966	59714	66444
Below Normal/Conserv.			35281	42801	51282	60878	68807	76287
Average/Advanced Cons			29903	35070	41145	49213	55908	61882
Below Normal/Advanced			34075	40054	47018	56310	64002	70821
(With Plumbing Code Only)								
Average/Conservation			31713	39231	48161	56991	64324	71641
Below Normal/Conserv.			36127	44969	55474	65494	74272	82734
Average/Advanced Cons			31212	38329	47293	56560	64323	71138
Below Normal/Advanced			35626	44068	54602	65263	74270	82231