

PERFORMANCE REPORT

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ENDANGERED AND THREATENED SPECIES CONSERVATION

Job No. 3.4: Central Texas Salamander Studies

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ABSTRACT

A wide array of biochemical and molecular methods are being used, in addition to morphological and morphometric work, to investigate genetic differentiation in Texas neotenic salamanders. The work to date has revealed definite patterns of divergence in this group that, in most cases, correspond to known geographic boundaries. It has become very clear that this group consists of more species than have previously been recognized, and several are in the process of being formally described. Implications for the overall taxonomy of this group are summarized. This, in turn, will provide a basis for conservation of critical habitats and biological diversity in the group, as has recently been done within the Balcones Canyonlands Conservation Plan.

PERFORMANCE REPORT

STATE: Texas PROJECT NO.: E-1-3
PROJECT TITLE: Endangered and Threatened Species Conservation.
PERIOD COVERED: May 1, 1991 through August 31, 1991.
JOB NUMBER: 3-4
JOB TITLE: Central Texas Salamander Studies.
JOB OBJECTIVE: To refine the distributional boundaries and taxonomic status of central Texas hemidactyliine plethodontid salamanders, and integrate this information with ecological requirements and known habitat threats to constituent populations.

ACCOMPLISHMENTS

See attached report.

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Central Texas neotenic salamanders (*Eurycea* and *Typhlomolge*):

Taxonomic status, relationships, distribution,
and genetic differentiation

Section 6 Interim Report

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INTRODUCTION AND BACKGROUND

The Edwards Plateau region of Central Texas is a center of biological diversity, the result of its location, topography, and limestone composition. The karst formations of the area contain many reliable springs and underground water systems that represent isolated pockets of habitat for numerous aquatic organisms. These include salamanders of the genera *Eurycea* and *Typhlomolge* (Plethodontidae: Plethodontinae: Hemidactyliini), all of which are unique to the region. In this interim report, we will discuss the taxonomic status and distributions of the various populations of these salamanders in central Texas, based on ongoing studies of genetic differentiation in the group that are being funded under the Section 6 cooperative agreement between the Texas Parks and Wildlife Department and the U.S. Fish and Wildlife Service.

As detailed in an earlier report (Chippindale et al. 1990), the number of species and genera of plethodontid salamanders in the Edwards Plateau region is unknown. Almost all populations of Texas salamanders assigned to the genus *Eurycea*, and both populations assigned to *Typhlomolge*, are neotenic i.e. retain an aquatic larval body form despite the attainment of reproductive maturity. Thus, individuals in most populations exhibit a "generalized" larval morphology which may be very similar among populations regardless of the degree of genetic divergence. Based on these morphological similarities, most spring-dwelling populations of *Eurycea* throughout the Edwards Plateau region have been assigned to a single species, *E. neotenes* (e.g. Sweet 1978, 1982). Our work to date (see below) clearly shows that "*E. neotenes*" consists of at least several species that exhibit high levels of genetic and/or morphological differentiation. Some of these undescribed species occur in areas that are under serious and immediate threat from development and aquifer depletion; their existence would not have been recognized without the use of biochemical and molecular systematic techniques.

Relationships of subterranean populations of neotenic salamanders in central Texas have also been problematic. The many underground populations of *Eurycea* show varying degrees of cave-associated morphology, and numerous instances of parallel morphological evolution appear to have occurred in the cave-dwelling members of the group (see also Sweet 1978, 1984). This has led to much controversy regarding the taxonomy of the group (Mitchell and Reddell 1965, Mitchell and Smith 1972,

Sweet 1978, 1984, Potter and Sweet 1981). At issue are recognition of the genus *Typhlomolge* as distinct from the Texas *Eurycea*, and the status and relationships of the various cave populations. Our molecular studies are shedding considerable light on these problems, as we will detail below.

Prior to our work, the following taxa of Texas neotenic salamanders have been recognized:

Eurycea neotenes Bishop and Wright, 1937. As currently recognized, this species ranges from Bell Co. in the northeastern part of the plateau (Balcones Fault Zone) region to Val Verde Co. in the southwest, occurring in both surface springs and caves. Evidence that this species is actually composed of several is provided in this report (see "New species, other taxonomic conclusions, and recommendations").

E. nana Bishop, 1941. Found at San Marcos ("Aquarena") Springs in Hays Co. Sweet (1978) suggested on the basis of morphology that a population at Comal Springs, Comal Co. might also represent this species, but did not elaborate. Our work provides strong evidence that the population of salamanders at San Marcos is distinct from all others and is not conspecific with the population at Comal Springs (see "Status and distribution of *Eurycea nana*" for details).

E. tridentifera Mitchell and Reddell, 1965. Troglotic (cave-dwelling) species that occurs in caves of the "Cibolo sinkhole plain" region of Comal, Kendall, and Bexar counties. Six localities were previously known (Sweet 1977); several others have since been discovered (see Appendix 1).

E. latitans Smith and Potter, 1946. Morphologically variable form that occurs in the Cascade Caverns system of Kendall Co. May represent hybrids between *E. neotenes* and *E. tridentifera* (Sweet 1984). We will investigate the status of this form in the present research.

E. troglodytes Baker, 1957. Known from Valdina Farms Sinkhole, Medina Co.; may represent hybrids between *E. neotenes* and *E. tridentifera* (Sweet 1984). May be extinct due to massive flooding of the cave by a dam project in the late 1970's; the cave fauna of this major sinkhole system appears to have been destroyed by this incident (G. Veni, pers. comm.).

E. sp. Found in the subterranean system beneath Barton Springs in Travis Co., this population was recognized as a distinct species on the basis of morphology by Sweet (1978, 1984) but has not yet been formally described. Our biochemical work (see below) supports Sweet's conclusions. Degradation of water quality in the Barton Springs watershed may pose a serious threat to the continued survival of this species.

Typhlomolge rathbuni Stejneger, 1896. Extreme troglobite from the San Marcos Pool of the Edwards Aquifer. Little is known of its distribution, habits, or relationships; others (e.g. Mitchell and Reddell 1965, Mitchell and Smith 1972) have suggested that this species belongs in the genus *Eurycea*, and our work supports this view.

T. robusta Potter and Sweet, 1981. Known from one specimen collected in 1951 from underneath the bed of the Blanco River near San Marcos; prospects for collecting additional specimens of this species are uncertain.

PROGRESS TO DATE

We are currently using a wide array of biochemical and molecular methods to investigate genetic differentiation in the Texas neotenic salamanders, in addition to morphological and morphometric work. This work is both time-consuming and broad in scope, and the results presented here must be regarded as preliminary, as these studies are ongoing. The work to date has revealed definite patterns of divergence in this group that, in most cases, correspond to known geographic boundaries. It has become very clear that this group consists of more species than have previously been recognized; here we will summarize the work to date and its implications for the taxonomy of the Texas neotenic salamanders. This, in turn, will provide a basis for conservation of critical habitats and biological diversity in the group.

Field work

We have obtained specimens from 52 localities in 14 of the 16 counties in which neotenic salamanders are known to occur in central Texas. We have not yet been

able to obtain specimens from the two known localities in Val Verde Co. (San Felipe Springs and Four Mile Cave; see Sweet 1978, 1982) despite several attempts. We have not yet obtained specimens from Medina Co., but plan to do so. One other county (Kinney Co.) may also harbor neotenic salamanders, but no localities are known. We have discovered many new localities in the course of our work, and others have been brought to our attention, in particular by cavers. New and previously unreported localities are listed in Appendix 1; these include only new localities discovered since our last interim report (fall 1990), plus little-known salamander caves not listed in that report.

Among the most noteworthy new localities are those from the Jollyville Plateau region northwest of Austin (Barlow Hollow Spring, Balcones Park Spring, Bull Creek Springs), the Cedar Park area (Audubon Springs, Testudo Tube, Buttercup River Cave), and the Georgetown area (Crockett Garden Spring, Cedar Breaks Trail Spring, Buford Hollow Springs). As detailed below, salamanders from these areas northeast of the Colorado River clearly represent at least one new species, and thorough knowledge of the geographic distribution of populations in this region is crucial to their protection.

Allozyme work

Allozyme electrophoresis involves placing samples of tissue homogenates in a starch gel and subjecting the gel to an electrical current. This causes the various proteins in the sample to move through the starch matrix at varying rates, depending on the proteins' net charge and conformation. Allelic variants of the proteins can be identified by staining the gel for specific enzymatic proteins and determining the relative mobilities of the chosen proteins for samples that represent different individuals and taxa. The basis of these differences in mobility is assumed to be genetic, and thus provides a measure of genetic differentiation among individuals, populations, and/or taxa. Details of these methods are given in Murphy et al. (1990); specific enzyme-encoding loci and electrophoretic conditions used in our study of Texas neotenic salamanders are listed in Chippindale et al. (1990).

This approach has proven extremely useful for identifying major subdivisions and groups among the Texas neotenic salamanders. So far, we have screened 26

enzyme-encoding loci for 232 individuals from 46 populations of these salamanders. Patterns of overall genetic similarity are shown in Figs. 5 - 7. Major points to note are the following: all of the populations from the "northern" region (northeast of the Colorado River) are strongly differentiated from those to the south. This high degree of genetic divergence suggests a long period of separation despite a high degree of morphological similarity between individuals from many populations in this region and those from the southern region. In fact, *Typhlomolge rathbuni*, despite its extremely distinctive morphology, is actually more similar genetically to other populations in the southern region than these southern populations are to those in the north. Also noteworthy is the division on the basis of overall similarity between the group of spring and cave populations in the eastern portion of the southern region, and those in the western portion of the southern region. This division corresponds to an apparent hiatus in the distribution of salamanders in the southern region (further field work is necessary to determine whether this break in the distribution is real or simply an artifact of accessibility of habitat to collectors). While more work is necessary, this does suggest that there may be at least two major, genetically distinct groups of populations of "*E. neotenes*" in the southern region. This is most evident in Fig. 7, which diagrams the pattern of genetic distances in the form of a "topographic map".

On the basis of overall similarity to other populations, both *T. rathbuni* and the San Marcos Springs population assigned to *E. nana* cluster outside of the other southern populations; this illustrates the genetic distinctiveness of each of these populations, consistent with their recognition as separate taxa. Note that the Comal Springs population that has sometimes been recognized as *E. nana* is genetically very distant from the San Marcos Springs population of *E. nana* and clusters in a separate subgroup of southern populations. As detailed below (see "Status and distribution of *Eurycea nana*"), the Comal Springs population also appears to share none of the many unique alleles that characterize the San Marcos population.

Various cave populations that are regarded as or have been thought to represent distinct species have proven, on the basis of allozymes, to be very similar genetically to nearby surface populations. This is evident for *E. tridentifera* which, although morphologically very distinct, clusters on the basis of genetic distances within the assemblage of geographically proximal populations of "*E. neotenes*". This does not mean that *E. tridentifera* should not be recognized as a separate species, but simply

illustrates the differing patterns of morphological versus molecular divergence in the group, and further suggests that there may be many more species present than previously thought.

The above analysis provides a measure of genetic similarities among populations and helps to identify major, potentially cohesive groupings of these salamanders. However, clustering based on overall similarity is likely not the best way to determine historical patterns of relationships. To investigate relationships among major groups of populations and taxa, we have employed parsimony methods of phylogeny reconstruction (see Swofford and Olsen 1990 for a discussion of these approaches). Because the computer programs available to carry out such analyses cannot practically handle large numbers of populations or taxa, we subdivided the Texas neotenic salamanders into several major groupings on the basis of apparent genetic cohesiveness and geographic distribution. We then coded the loci surveyed as characters and the alleles at these loci as states and carried out a branch-and-bound search using Swofford's (1990) PAUP program. The results are shown in Fig. 8. Although the degree of resolution is low, two major clades (groups of populations and/or taxa, within each of which the members are most closely related to one another and share a common ancestor) are evident. These correspond to the two major groups identified in the phenetic (similarity, or genetic distance-based) analysis: "northern" versus "southern". Again, *T. rathbuni* falls outside the other southern populations but still appears to most closely related to this group, while the deepest split in the tree corresponds exactly to the "north vs. south" divide of the Colorado River. This further supports recognition of the northern group of populations as at least one distinct species, as well as placement of *T. rathbuni* in the genus *Eurycea*.

Ribosomal DNA

Allozyme electrophoresis has proven extremely useful in identifying major divisions in the Texas neotenic salamanders. However, in some cases, allozymes alone have not allowed full resolution of relationships and species boundaries in the group. It is also desirable to be able to compare conclusions based on one data set, such as allozymes, to those based on one or more independent data set(s). For these reasons, we are currently carrying out investigations of DNA restriction site

variation in both the nuclear ribosomal genes and the mitochondrial genome for the Texas neotenic salamanders.

Ribosomal DNA (rDNA) encodes the RNA that makes up a portion of the ribosomes in all cells. In the nuclear genome, multiple copies of the rDNA repeat unit are present. It is composed of several subregions that span varying degrees of evolutionary conservatism, from regions that evolve extremely slowly and are useful primarily for investigating ancient divergences to those that evolve very rapidly and can be used to study differentiation at the within-species level (see Hillis and Davis 1988). We have chosen to investigate restriction site variation throughout the transcribed region of the rDNA repeat (the nontranscribed spacer region has proven to be extremely variable, even within individuals, and is thus relatively intractable for use in this study).

This work involves isolating total cellular DNA from salamander tissues, and then literally cutting up the DNA with bacterial enzymes (restriction enzymes) that recognize specific sequences of four, six, or eight base-pairs. Since DNA consists of four different bases (A,G,T,C), the chances of a particular sequence of bases occurring at any given place in the DNA are very low, and diminish exponentially with increasing length of recognition sequence. Shared restriction site gains can provide evidence of relationship among populations or taxa, since the probability of two taxa having gained a given restriction site independently are very low. To visualize and characterize restriction site variation in salamander rDNA, we use methods of southern blotting and hybridization detailed in Dowling et al. (1990), and apply a series of radioactively labelled DNA probes that are specific to different regions of the rDNA repeat unit to determine ("map") the precise locations of particular restriction sites. After a great deal of experimentation with techniques, we have refined these methods so that they work reliably with the Texas salamanders.

Results of this approach are preliminary so far, but it is clear that there is substantial phylogenetic information present in the rDNA of this group. In particular, sites recognized by the enzymes Bcl I, Bgl I, BamH I, EcoR V, EcoN I, and Pst I have proven to be variable within the transcribed region of the ribosomal repeat unit in a survey of approximately 30 mainly six-base cutting enzymes for approximately 20 populations and taxa. We will survey additional individuals from many populations for variation using these enzymes; this will provide a separate data set

for the nuclear genome to test our allozyme-based hypotheses of relationships in the group.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) represents a genome distinct from that of the nucleus, one that evolves relatively rapidly on average and is not subject to recombination (it is passed on intact from female to offspring with no contribution of mitochondrial genetic material from the male). mtDNA has proven to be an extremely useful molecule for determining relationships, biogeographic history, and species boundaries in closely related populations and taxa (see review by Moritz et al. 1987). Since it evolves independently from the nuclear genome, inferences of relationships based on mtDNA can be compared to and in some cases be used to test those based on nuclear markers. mtDNA appears ideal for investigation of fine-scale differentiation and divergence in the Texas neotenic salamanders, and we are currently using this approach in our work on the group.

Since the salamanders in our study are relatively small, it has proven impossible to obtain enough tissue to extract usable quantities of mtDNA to work with directly. This has necessitated an alternate strategy: we have been able to use mtDNA-specific probes to identify fragments of restriction enzyme cut mtDNA on southern blots of total cut cellular DNA (for details of this general approach, see Sites and Davis 1989). Initial trials have involved amplifying a portion of the mitochondrial 12S rDNA gene via PCR (see Hillis et al. 1990 and references therein), then producing a radioactive probe from this DNA by primer extension (see Sambrook et al. 1990). This has allowed us to visualize fragments of mtDNA that contain all or part of the amplified 12S region. This has revealed a high degree of restriction site variation in mtDNA which so far (based on very preliminary observations) appears to correspond well to that revealed by allozymes. However, what we have been able to visualize so far represents only a small portion of the mitochondrial genome. To see the whole picture, we are working to clone the entire mitochondrial genome from a representative of this group for use as a probe, and also develop more region-specific probes for mapping restriction sites. All of this takes a great deal of trial and error, but preliminary results are encouraging and we expect to be able to gather substantial mtDNA data in the near future.

Genome size measurement

In collaboration with L.A. Lowcock (University of Toronto), we are investigating genome size variation in the Texas neotenic salamanders using flow cytometric methods for determination of total nuclear DNA content. While this research is not primarily taxonomic in nature, it has revealed a strong pattern of differentiation in genome size that distinguishes two major groups in the Texas assemblage. Surveys of numerous individuals from both the "southern" and "northern" groups of populations of "*E. neotenes*" have shown that individuals from the northern populations have, on average, approximately 13% more DNA per nucleus than those from the southern group (the difference is highly significant). This genome size division corresponds precisely to the deepest division in "*neotenes*" based on allozymes, and provides further evidence of the major genetic split in the group.

Morphometric work

We have used discriminant function analysis to obtain an initial picture of morphometric variation among populations of Texas *Eurycea*, using 11 measures of external body proportions. This multivariate approach involves combining the original set of variables into an array of new variables that maximize the separation among previously designated groups (here, populations/taxa) in multidimensional space. This technique can serve to identify morphological differences that are the result of factors such as overall shape and contrasts among body proportions (e.g. head width versus body length). Using the discriminant functions, individuals designated as "unknowns" can then be tested for placement in the previously characterized groups to obtain a measure of the distinctiveness of the various groups. This approach was used by Sweet (1978, 1984) to assess morphological variation among cave-dwelling populations of the Texas *Eurycea*.

Results of an initial discriminant analysis in which salamanders from surface populations were assigned to the groups "northern", "southeastern", "southwestern", "Comal Springs", "San Marcos Springs" and "Salado" revealed considerable overlap among several of these groups in external morphology using the 11 measurements. This is illustrated in Fig. 9, in which group centroids and scores for outlying individuals are plotted with respect to the first and second canonical variate axes that result from the analysis. However, members of the

Salado Springs population, a geographically peripheral locality in Bell Co., are very distinct from all others even upon cursory examination, and are demonstrably so on the basis of discriminant analysis (Fig. 9). This isolated lineage is genetically very similar to other northern populations but has undergone substantial morphological differentiation.

In this analysis, individuals from Comal Springs showed considerable morphometric overlap with other populations currently assigned to *E. neotenes*. However, *E. nana* from San Marcos Springs were distinguishable from Comal Springs animals (Fig. 9). As is the case for the Salado population, this can also be discerned by eye; San Marcos animals appear (to us) more gracile than those from Comal Springs.

The discriminant analysis approach provides a useful measure of overall morphological (morphometric) similarity among populations and groups of populations. We are currently working to expand this aspect of the study, and are gathering measurement data on many more specimens, particularly representatives of the northern group. This should reveal morphological features that could be used to distinguish among populations, major groups of populations, and taxa.

Status and distribution of *Eurycea nana*

The status of the Comal Springs population of *Eurycea* is currently at issue, as noted earlier in this report. Sweet (1978, p. 131) stated that, "The population inhabiting San Marcos Springs has been described as *Eurycea nana* (Bishop, 1941), and is generally recognized as valid; the population which inhabits Comal Springs, some 27 km to the southwest, is very similar to *E. nana* and is probably conspecific...". However, Sweet (1978) provided no further details, and his assignment of the Comal Springs population to *E. nana* should be regarded as tentative. The tendency in recent years has been to treat the Comal Springs population as conspecific with that at San Marcos, not an unreasonable approach given the previous lack of evidence to the contrary, the relatively close proximity of these populations, and the general uncertainty regarding the taxonomy of Texas neotenic *Eurycea*. However, our initial investigations of allozyme variation in the group (Chippindale et al. 1990) suggested strongly that the Comal Springs population is genetically distinct from that at San Marcos. Allozyme and morphometric data

collected since that time support the same conclusion: the San Marcos population appears to represent a distinct lineage that is genetically very divergent from all other Texas neotenic salamanders that we have examined to date, including the Comal Springs population. [Note that our Comal springs specimens were collected at the same sites that Sweet visited (Sweet, in litt. Aug. 4, 1983)].

Appendix 2 provides genotype frequencies for the eight enzyme-encoding loci (of 26 surveyed) that differentiate the San Marcos population from the Comal Springs population. These are based on 11 individuals from Comal Springs and 12 from San Marcos Springs. Seven of these loci exhibit mutually exclusive allelic composition and/or fixed allelic differences between populations; Peptidase-A is probably also fixed different between populations, but this locus has proven difficult to interpret in the San Marcos animals, so results for this locus should be regarded as preliminary. No genetic variation has been observed in the San Marcos population based on allozymes: all 12 individuals from San Marcos surveyed were homozygous for all loci that could be resolved. Of the seven to eight loci that differentiate the two populations, five to six (including Pep-A) appear to be fixed for alleles unique to the San Marcos population (we have not found these alleles elsewhere among Texas neotenic salamanders). We detected no unique alleles in the Comal Springs population, although the "e" allele at the Aconitate hydratase 1 locus has otherwise been observed only in *T. rathbuni*.

As noted above (see "Morphometric work"), we have also been able to distinguish between individuals from the San Marcos and Comal Springs populations in preliminary discriminant function analyses, using measures of external body proportions. It is especially noteworthy that six of the 12 Comal Springs animals used in these analyses were specimens from the Texas Natural History Collection (Texas Memorial Museum); these were among the specimens examined by Sweet (1978), who suggested the Comal Springs population might be conspecific with the population at San Marcos Springs. We will carry out additional morphological and morphometric studies in an attempt to quantify specific points of difference between the two populations.

One alternative to our conclusions has been suggested by Sweet (pers. comm.): perhaps both *E. nana* and a member of the *E. neotenes* group occur at Comal Springs, and only one of these species was sampled in this study. While this

possibility cannot be dismissed, we think that this is unlikely, particularly given our ability to discriminate morphometrically between specimens from San Marcos and those collected both recently and well over a decade ago from Comal Springs. We will, however, examine additional specimens from both localities for morphological and morphometric variation. Another potential test of this hypothesis would be to amplify and sequence DNA from San Marcos animals and some of the Comal Springs specimens examined by Sweet (1978), using PCR methods (using this approach, it is possible to make multiple copies of specific DNA sequences from even preserved specimens under some circumstances). If consistent sequence differences can be found, this could provide a further test of Sweet's hypothesis. We will investigate the feasibility of this approach as part of our continuing work on Texas *Eurycea*.

Given the existing information, the weight of evidence clearly favors recognition of the San Marcos population as a distinct lineage, and it appears that this population alone (from which the type specimen of *E. nana* was collected) should be regarded as *E. nana*. The Comal Springs population appears to be one of the many populations that currently fall under the heading of "*E. neotenes*". However, like so many populations assigned to this species, it is almost certainly isolated from gene flow with other populations and could therefore be regarded as a distinct lineage (recall also that is also genetically identifiable based on the Aco-1 allele shared only with *T. rathbuni*). We are carrying out further studies to better determine the appropriate taxonomic standing of this population and others. This large and apparently thriving population would presumably be extirpated if Comal Springs were to go dry for an extended period of time. This appears to be a real threat, and should be addressed if this isolated population of uncertain taxonomic status is to survive.

New species, other taxonomic conclusions, and recommendations

Although the work described here is preliminary, the evidence that we have gathered to date has allowed us to identify three populations or groups of populations that deserve recognition as new species. These are the Barton Springs population, also regarded by Sweet (1978, 1984) as a distinct species on the basis of morphology; the group of populations northeast of the Colorado River that have

proven to be highly divergent from all others on the basis of allozymes and genome size; and the Salado population, which is genetically very similar to other northern populations, but is morphologically very distinct and is clearly isolated from gene flow. We are currently preparing descriptions of these new species, and we believe that all three deserve the protection that is afforded by listing at the federal and state levels. The status of salamanders in the Cedar Park caves and the Georgetown region (Williamson Co.) is currently uncertain, and further biochemical and molecular study is necessary. Both these groups of populations are clearly very closely related to those in the Jollyville Plateau and Round Rock areas, and the most reasonable course of action at present appears to be to treat them as conspecific, on the understanding that they may later be found to represent additional species.

Although no data are available regarding the effects of specific pollutants on populations of Texas neotenic salamanders, it seems likely that any substantial degradation of water quality could be detrimental to the survival of populations of these amphibians, especially since they are aquatic and have apparently evolved under conditions of reliable spring flow and constancy of water chemistry. Thus, the increase in pollution that appears to be occurring in the Barton Springs system may well pose a serious threat to survival of the undescribed species that occurs there. Sweet (1978, 1982) documented the very specific conditions of spring location and flow that are required by surface-dwelling populations of Texas neotenic salamanders. Given the small size and fragmented nature of the aquifers and their associated recharge zones in the region north of the Colorado River, special attention must be paid to development and uses of water that may have an impact on these underground water systems; otherwise springs upon which the undescribed northern species depend may cease to flow reliably. This already appears to have happened at one Travis Co. locality from which *Eurycea* were known (MacDonald Well Spring). The rapid pace of development in this northern region poses other threats to salamander populations: for example, the population at Krienke Spring (Williamson Co.) has apparently been eliminated by quarrying at the spring site (Sweet 1978), and the entrance to Salamander Cave in northwest Austin, which contained a population of morphologically very distinct *Eurycea*, has apparently been covered by a road or other development (J. Reddell, pers. comm.).

Continued work will allow us to identify additional populations and groups of populations that represent genetically distinct lineages in need of protection. Our

results so far demonstrate substantial fragmentation and genetic diversity in the Texas neotenic *Eurycea*, and it is essential that this diversity be recognized in time for adequate conservation measures to be implemented. Such measures, which would guarantee preservation of water quality and quantity, should ultimately prove beneficial to all organisms, including humans, that depend upon this resource.

FUTURE WORK

We have collected material from most of the surface populations that are necessary to investigate genetic variation in the Texas neotenic salamanders on a broad scale. However, there are several important cave localities that remain to be visited. Especially critical are caves in the Comal/Kendall Co. region, as the status of *E. tridentifera* and especially "*E. latitans*" require further investigation. Our work so far indicates that the populations in Honey Creek Cave and Badweather Pit both represent *E. tridentifera*; this is consistent with Sweet's (1978, 1984) conclusions based on morphology. However, more localities need to be sampled to determine the genetic cohesiveness of this subterranean species and to test Sweet's hypothesis that *E. latitans* is an invalid taxon derived from hybridization between *E. tridentifera* and *E. neotenes*. Other important caves to visit are those in the Cedar Park region, since the material that we have is limited, and Windmill Cave in Medina Co. Another important region to sample will be the area between the "southeastern" and "southwestern" groups of spring and cave populations shown in Fig. 7; we need to determine whether the apparent hiatus in the distribution of salamanders in this area, and the accompanying division on the basis of genetic distance, are real. If so, this could provide the basis for recognizing additional species in the *E. neotenes* group. Another important region to investigate will be northeastern Kinney Co., where *Eurycea* are likely to occur but have not yet been reported.

With respect to laboratory work, it will be important to pursue the mitochondrial DNA investigations, as this approach is likely to yield much more insight into biogeography, differentiation, and species boundaries in the group. Continued allozyme and ribosomal DNA restriction site studies should also prove very informative, and are in progress.

The preliminary results reported here provide many new insights into the relationships and complex biogeographic history of the Texas neotenic salamanders, *Eurycea* and *Typhlomolge*. These salamanders are restricted to "islands" of aquatic habitat in the Edwards Plateau region, and their history of fragmentation and peripheral isolation has led to substantial genetic differentiation in the group. This process has resulted in the occurrence of numerous genetically distinct lineages in the region, and our work indicates that there are probably many undescribed species involved. Recognition and characterization of the genetic diversity in the group is essential if the many species of central Texas neotenic salamanders are to be preserved. Our work to date has provided the basis for protecting some of these geographically restricted species; further investigation will lay the foundation for additional conservation measures necessary to protect genetically distinct populations of these salamanders and their fragile spring and cave habitats.

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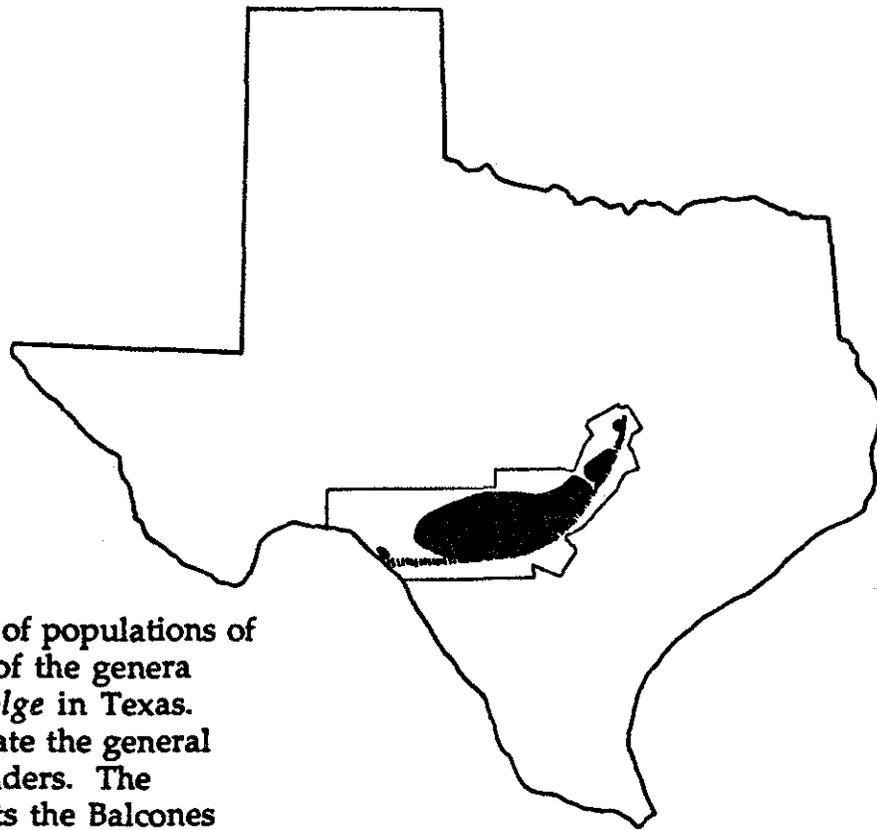


Figure 1. Distribution of populations of neotenic salamanders of the genera *Eurycea* and *Typhlomolge* in Texas. Blackened areas indicate the general range of these salamanders. The hatched line represents the Balcones Escarpment, which delineates the southern and eastern margins of the Edwards Plateau. The angular outline represents counties where these salamanders are known or thought to occur (*Typhlomolge* is thought to be restricted to Hays Co.).

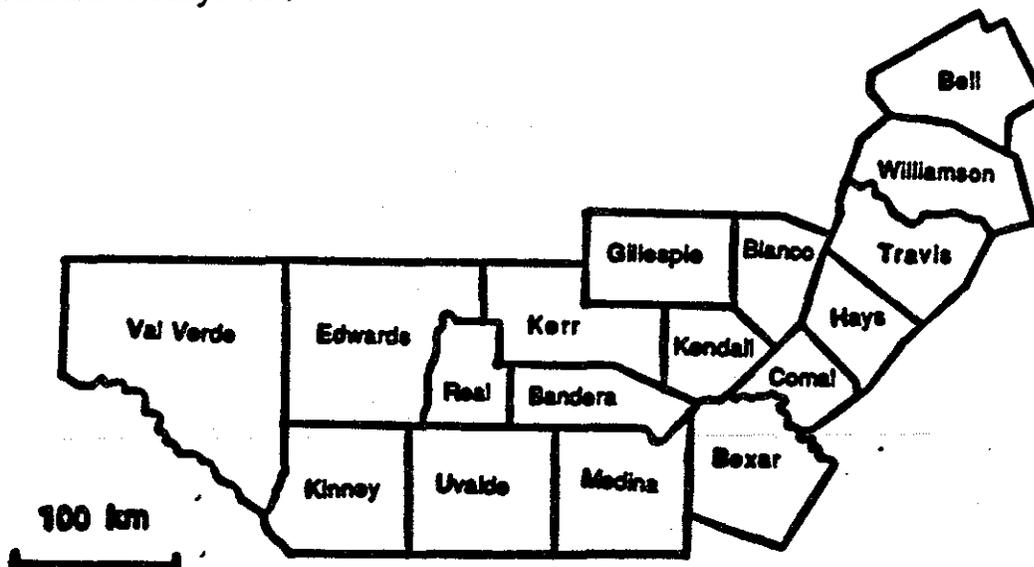


Figure 2. Counties in the Edwards Plateau region of central Texas that are known or thought (Kinney Co.) to be inhabited by salamanders of the genera *Eurycea* and *Typhlomolge* (the latter is thought to be restricted to Hays Co.). Note that outline of counties corresponds to that in Fig. 1.

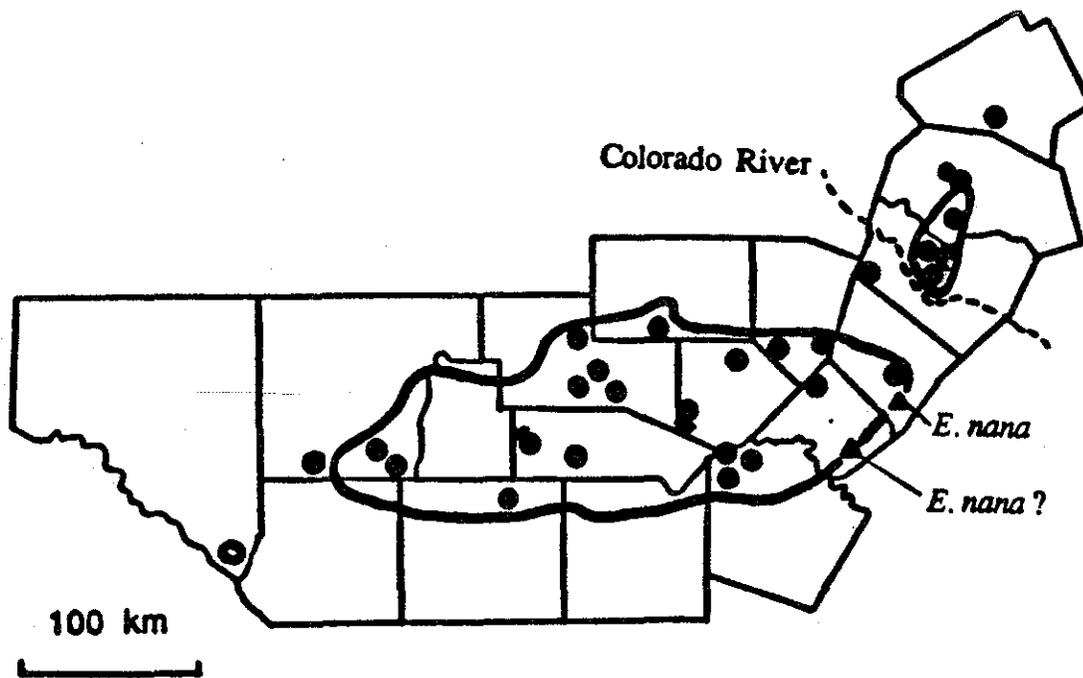


Figure 3. Surface spring localities in which Texas *Eurycea* have been collected in this study. The outlined area represents the known range of populations of *Eurycea* in the Edwards Plateau region, based on Sweet (1982). All populations indicated by blackened circles are currently allocated to *E. neotenes*, or represent newly discovered populations with no formal taxonomic status. The two populations indicated by black triangles have been assigned to *E. nana*; the more northern of the two represents San Marcos Springs (type locality for *E. nana*) and the more southern, Comal Springs (a questionable locality for this species – see text).

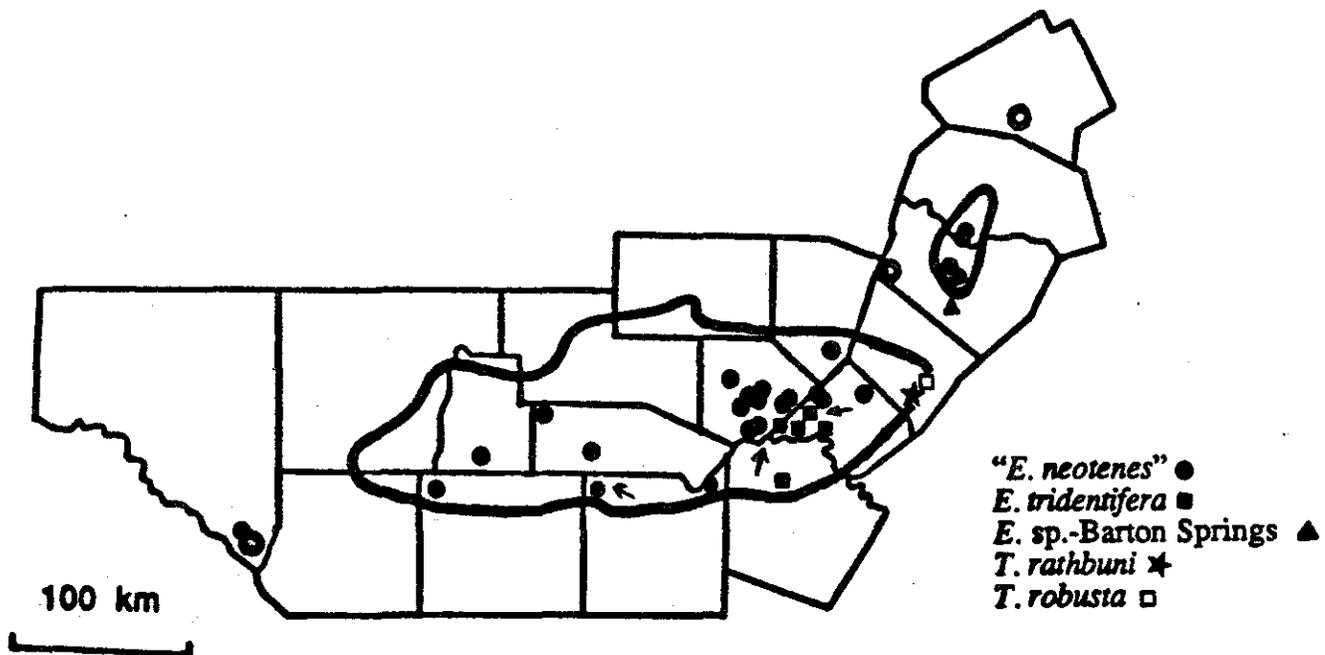


Figure 4. Distribution of subterranean populations of *Eurycea* and *Typhlomolge* in central Texas. Symbols indicate populations that are currently thought to represent distinct species, based on morphology. Most other populations are currently assigned to *E. neotenes*; areas indicated by arrows are those in which hybridization between *E. neotenes* and *E. tridentifera* may occur (see Sweet 1982, 1984).

Figure 5. (following page) Phenetic (UPGMA) clustering of Rogers' genetic distances for Texas neotenic salamanders, based on allozyme data for 25 enzyme-encoding loci. Populations that are currently recognized as distinct species are designated by scientific name. *E. sp.* refers to the Barton Springs population; "*E. nana*" in the cluster of southeastern populations refers to the Comal Springs population that has sometimes been recognized as *E. nana*. Note that on the basis of genetic distance, the Comal Springs population clusters in a distinct group from the population of *E. nana* at San Marcos Springs. The remaining populations have previously been assigned to *E. neotenes* or are newly discovered populations that currently have no formal taxonomic status. Populations designated by number are identified in the accompanying list of localities. North refers to the region northeast of the Colorado River; southeast refers to the eastern portion of the region south of the Colorado River; and southwest refers to the western portion of the region south of the Colorado River (see Fig. 7 for more detail).

Figure 6 (second following page). The same phenogram as in Fig. 5, with cave populations indicated in bold. This demonstrates that, despite the diversity of morphologies exhibited by the cave populations, they do cluster as most similar to nearby surface populations within each major region.

Figure 5

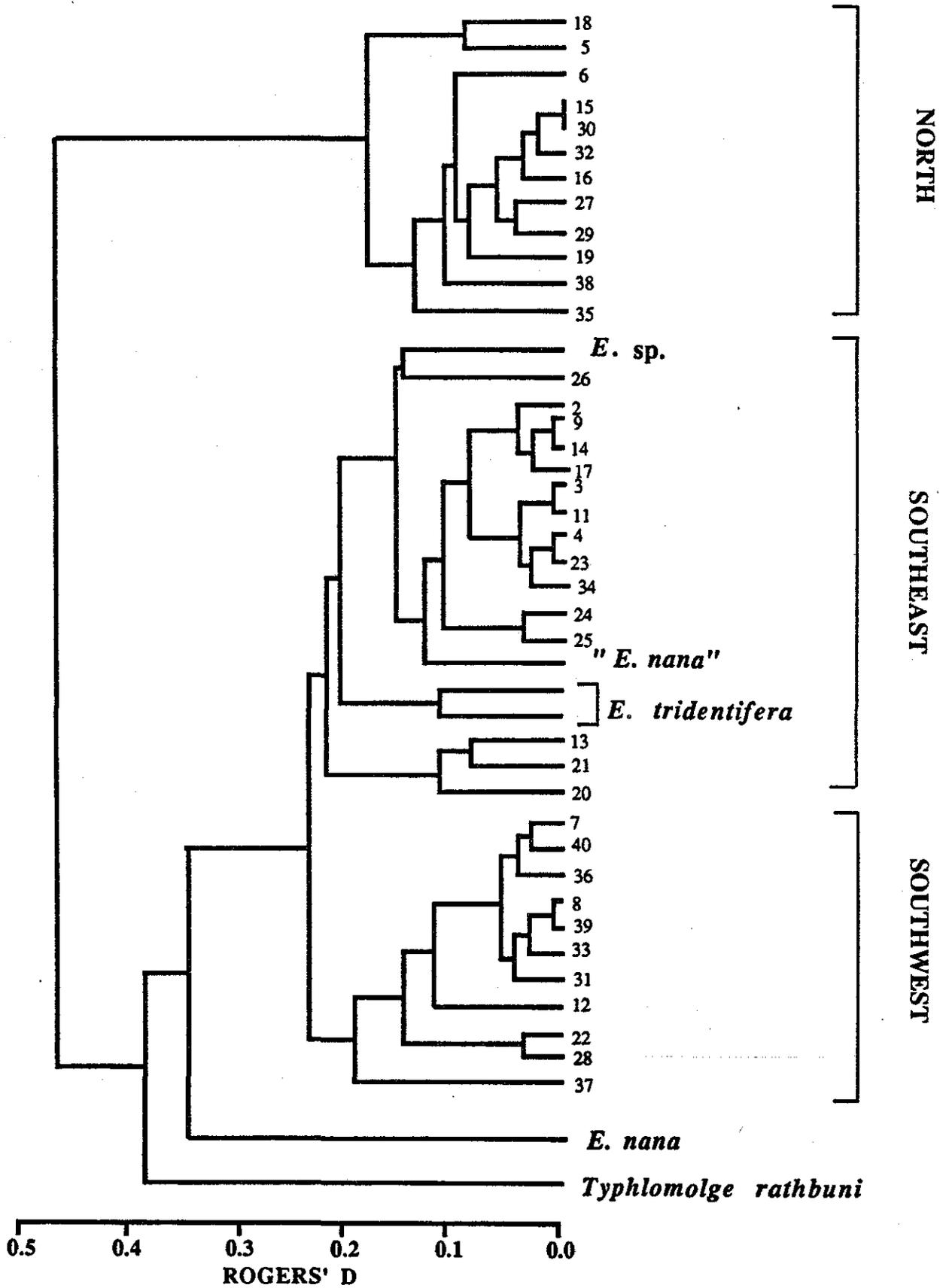
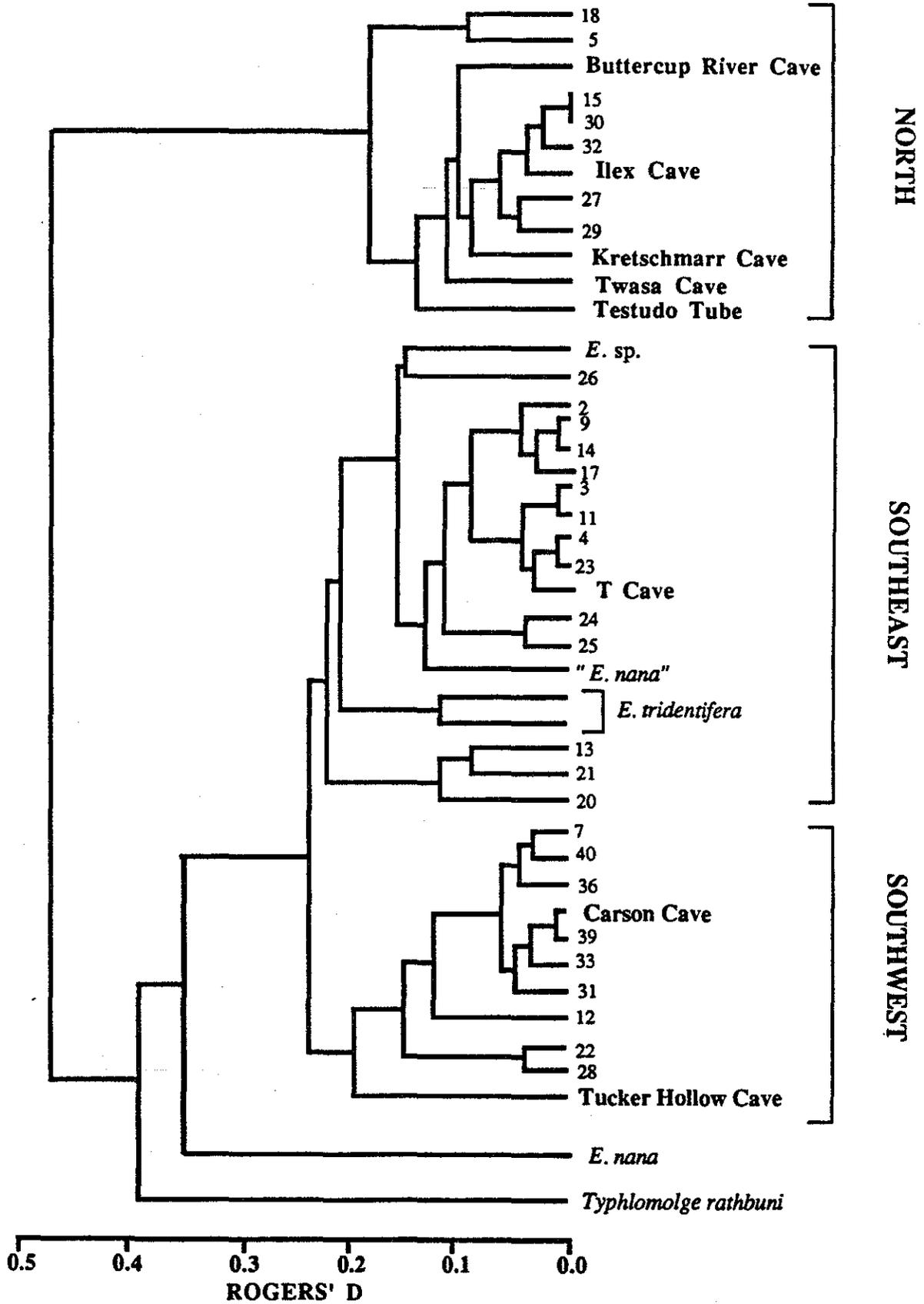


Figure 6



KEY TO NUMBERED POPULATIONS IN FIGURES 5 AND 6

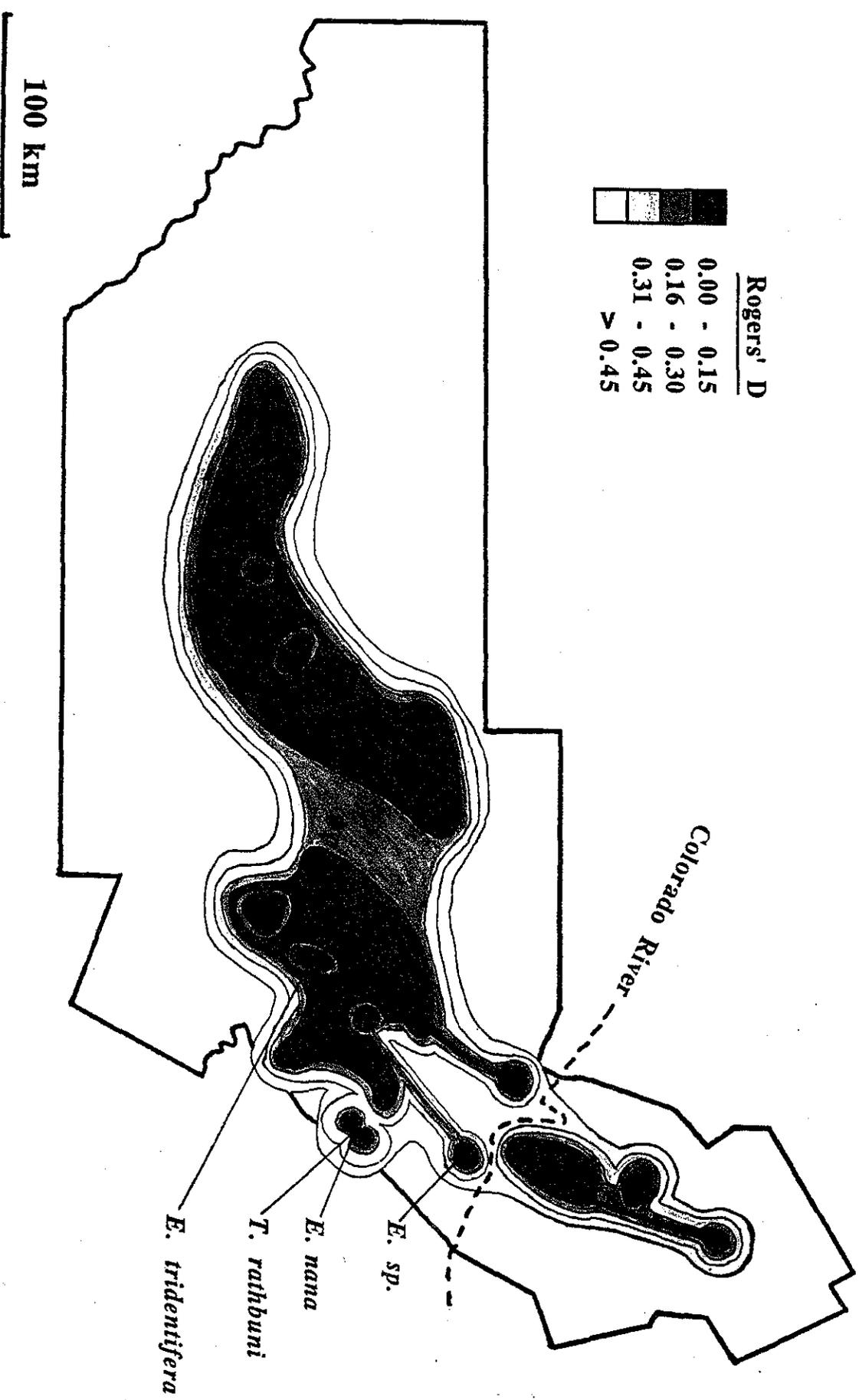
1. Barton Springs (Travis Co.; listed as *E. sp.* on figures)
2. Bear Creek Spring (Kendall Co.)
3. Blanco Spring (Blanco Co.)
4. Boardhouse Spring (Blanco Co.)
5. Bruford Hollow Spring (Williamson Co.)
6. Buttercup River Cave (Williamson Co.)
7. Camp Mystic Spring (Kerr Co.)
8. Carson Cave (Uvalde Co.)
9. Cibolo Creek tributary spring (Kendall Co.)
10. Comal Springs (Comal Co.; listed as "*E. nana*" on figures)
11. Fern Bank Spring (Hays Co.)
12. Fessenden Spring (Kerr Co.)
13. Helotes Creek Spring (Bexar Co.)
14. Honey Creek Cave, spring outside cave (Comal Co.)
15. Horsethief Hollow Spring (Travis Co.)
16. Ilex Cave (Williamson Co.)
17. Kneedeep Cave Spring (Kendall Co.)
18. Knight (=Crockett Garden) Spring (Williamson Co.)
19. Kretschmarr Salamander Cave (Travis Co.)
20. Leon Springs (Bexar Co.)
21. Mueller's Spring (Bexar Co.)
22. Murphy's Spring (= North Fork Sabinal River tributary spring) (Bandera Co.)
23. Peavey's Springs (Blanco Co.)
24. Pedernales Spring #1 (Travis Co.)
25. Pedernales Spring #2 (Travis Co.)
26. Rebecca Creek Spring (Comal Co.)
27. Round Rock (=Brushy Creek) Spring (Williamson Co.)
28. Sabinal Canyon Spring (Bandera Co.)
29. Salado Springs (Bell Co.)
30. Schlumberger Spring (Travis Co.)
31. Smith's Spring (Edwards Co.)
32. Stillhouse Hollow Spring (Travis Co.)
33. Sutherland Hollow Spring (Bandera Co.)
34. T Cave (Blanco Co.)

35. Testudo Tube (=Turtle Trot Cave, =Tortuga Cave) (Williamson Co.)
36. Trough Springs (Gillespie Co.)
37. Tucker Hollow Cave (Real Co.)
38. Twasa Cave (Williamson Co.)
39. West Nueces Spring (Edwards Co.)
40. Wetback Spring (Uvalde Co.)
41. Honey Creek Cave (Comal Co.) -- *E. tridentifera*
42. Badweather Pit (Comal Co.) -- *E. tridentifera*
43. San Marcos Springs (Comal Co.) -- *E. nana*
44. Spring Lake artesian outflow, Ezell's Cave, Rattlesnake Cave (Hays Co.) --*T. rathbuni*

Populations/taxa 1 and 41 to 44 are designated by species name only (not number) in figures 5 and 6.

Precise locality data for most of these populations are on file with the Natural Heritage section of the Texas Parks and Wildlife Department. Locations of newly discovered populations are listed in Appendix 1.

Figure 7 (following page). "Topographic map" of Rogers' (1972) genetic distances among populations of Texas neotenic salamanders. This figure illustrates the information given in Figures 5 and 6 in map form. Concentric lines from the outside in (yellow to red) represent successively smaller genetic distances among populations, i.e. greater genetic similarity. Genetic distances have been arbitrarily divided into intervals of 0.15 units for the purposes of this analysis. Black circles represent spring populations sampled in this study; black squares represent cave populations. Populations not designated by scientific name are either currently assigned to *E. neotenes* or are of uncertain taxonomic status. "*E. sp.*" refers to the Barton Springs population. Angular outline indicates counties in which these salamanders occur in central Texas, as in Figures 1 and 2.



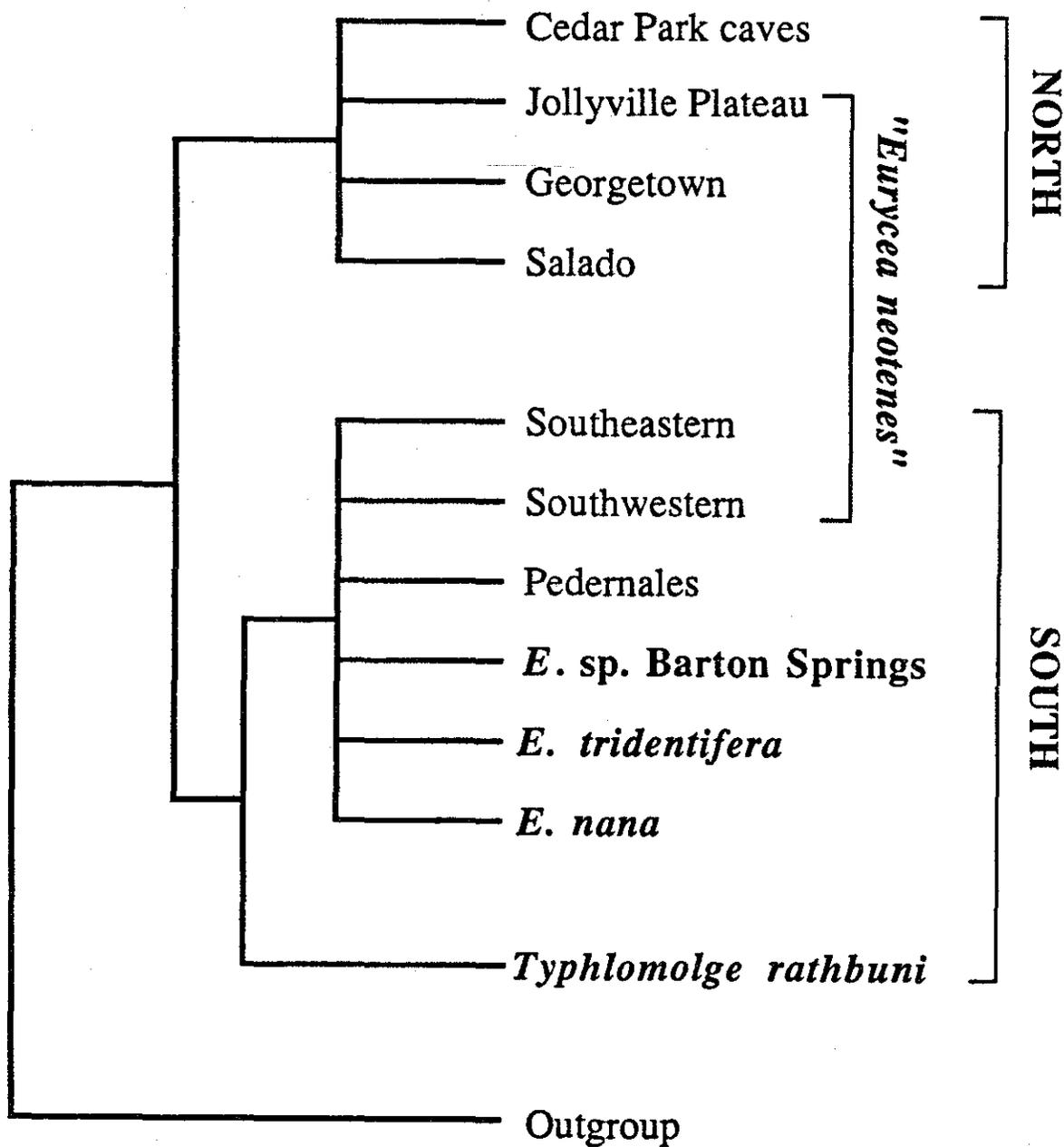


Figure 8. Relationships of major groups of populations and taxa of Texas neotenic salamanders, based on parsimony analysis of allozyme data. Note that the major division is between the group of populations to the north of the Colorado River and those to the south; groups of populations in each region that have previously been assigned to *E. neotenes* are indicated. The populations from the Pedernales and Cedar Park caves regions have been discovered recently and have no formal taxonomic status at present.

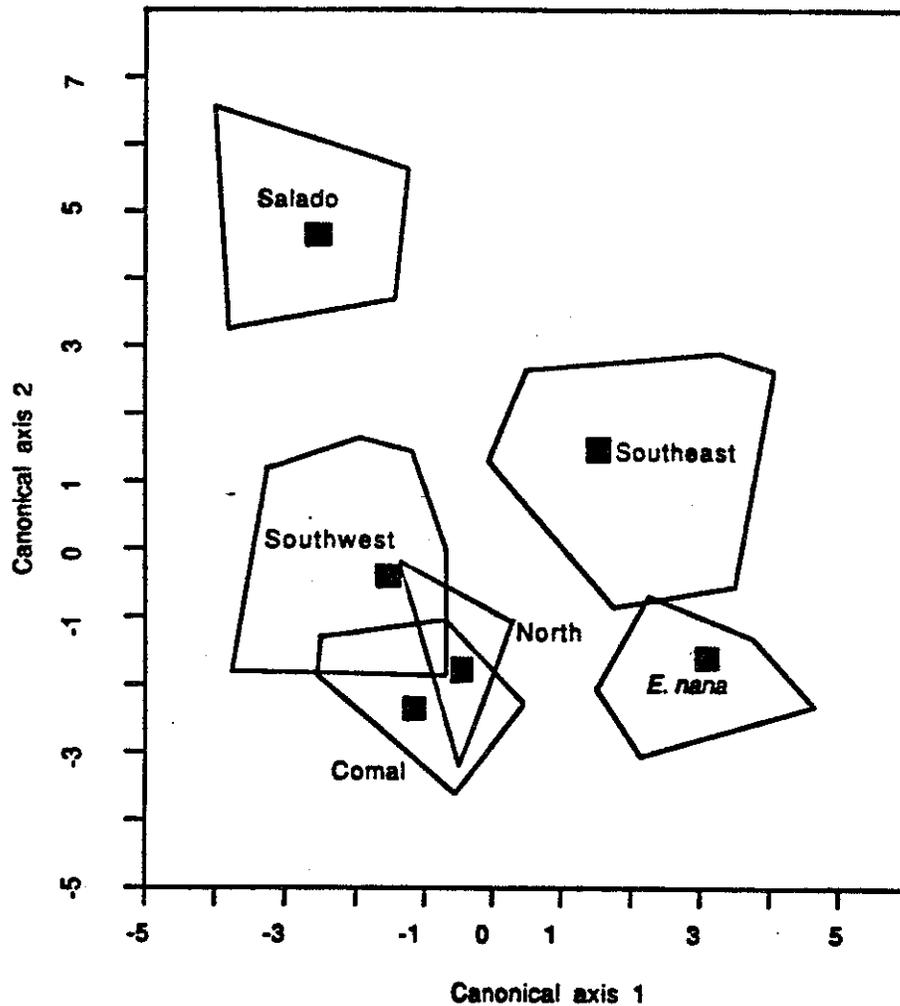


Figure 9. Plot of discriminant scores for samples of spring-dwelling populations of central Texas *Eurycea*, based on 11 morphometric variables. Group centroids are represented by squares; outlines around each centroid connect scores for individuals in each group that are furthest from the centroids. Members of all groups shown here have been assigned to *E. neotenes*, with the exceptions of *E. nana* (San Marcos Springs) and the Comal springs population that has sometimes been assigned to *E. nana*. The north, southeastern, and southwestern groups are composed of combined samples from each of these regions (see Figures 3 - 6). Note that the Salado population, a peripheral isolate from north of the Colorado River, shows a high degree of morphometric divergence from the other populations along the first two canonical axes. Note also that the Comal Springs population shows no morphometric overlap with *E. nana* from San Marcos Springs, although both populations have sometimes been regarded as conspecific. Sample sizes for this preliminary analysis ranged from four to 24 per group. The analysis was performed using the multigroup discriminant analysis (MDA) program in Biostat II (Pimentel and Smith 1986). Measures were log-transformed, and were as follows: eye diameter, anterior limb length, hind limb length, snout-vent length, axilla-groin distance, tail length, snout-gill distance, snout-gular fold distance, eye-gill distance, interocular distance, and head width.

APPENDIX 1: Locations of newly discovered, previously unpublished, and little-known populations of neotenic salamanders (*Eurycea* and *Typhlomolge*) in central Texas. This includes only populations that have been discovered since the time of our last interim report (Chippindale et al., Nov. 1990) or that were not reported there. See that report and TPWD files for details of other new localities that were discovered earlier in this study. Unless otherwise noted, we have obtained representative specimens from these localities.

Bexar Co.

Genesis Cave: Precise location to be determined; approximate location given in Veni (1988). Blind salamanders have been reported from a pool approximately 265 feet below the surface (D. Pearson, pers. comm.). No specimens are available at present. Due to the location and nature of the cave, it seems likely that these salamanders represent *E. tridentifera*, especially given the occurrence of this species at nearby Elm Springs Cave (Sweet 1978, Veni 1988).

Comal Co.

Ebert Cave: 29°45'06" N/98°23'28" W. A single salamander specimen from this cave has been provided by J. Reddell (Texas Memorial Museum). On the basis of morphology, this specimen appears to represent *E. tridentifera*, also known from nearby Kappelman Salamander Cave. We visited this locality in August 1991, but thorough exploration was impossible due to extremely bad air; additional exploration will be carried out in the near future.

Hays Co.

Grapevine Cave: Recently discovered; near Wimberley; precise location to be determined. A single small specimen is available, provided by J. Reddell. This specimen appears similar morphologically to surface populations of "*E. neotenes*".

Rattlesnake Cave: 29°54'07" N/97°55'17" W. *Typhlomolge rathbuni* have previously been reported from this locality (Russell 1976). Based on our investigations and Russell's, this species appears to be relatively abundant at this site. Proposed acquisition of the property by the TPWD may provide protection for this cave and the salamanders, and could permit the first detailed ecological studies to be carried out of this federally endangered species.

Kendall Co.

Detailed information on caves of this region is provided by Elliot (1985).

Kneedeep Cave Spring: 29°52'31" N/98°29'05" W, Guadalupe River State Park. Salamanders have been reported from inside this cave (Sweet 1978). We found no animals in the first several hundred meters of the cave, but were able to collect specimens of *neotenes*-like animals from the spring outflow of the cave.

Pfeiffer's Water Cave: 29°45'44" N/98°39'59" W, near Cascade Caverns. Blind salamanders have been reported from this locality (see Elliot 1985); we hope to visit this cave in the near future.

Sattler's Deep Pit: Sattler ranch near Spring Branch, approximately 29°55'32" N/98°26'27" W. A single specimen is said to be available (J. Reddell, pers. comm.), collected in summer or fall 1991. No further information is available at present, but we hope to visit and obtain additional material from this locality.

Schwarz Cave: 29°44'42" N/98°40'36" W, near Cascade Caverns. Salamanders have previously been reported to occur in this cave, and likely represent the form currently assigned to *E. latitans* (see Sweet 1978). J. Reddell has provided several preserved specimens of large, troglobitic salamanders from this locality, and further investigation is planned for the near future if possible.

Medina Co.

Windmill Cave: Precise location needs to be confirmed by visiting the site; cave is located in NW quarter of the Timber Creek quadrangle and is thought to be marked

on the map by a windmill. This cave has been investigated by cavers; specimens of troglobitic salamanders are reported to have been collected from this locality (J. Reddell, pers. comm.), but we have not yet been able to examine them. A visit to this cave is planned for the near future.

Travis Co.

Audubon Salamander Spring: 30°28'38" N/97°52'08" W.; **Baker Spring:** 30°28'48" N/ 97°52'06" W. Audubon conservation area near Cedar Park. *Eurycea* have been observed at these two springs on this property; none have been collected.

Balcones Park Spring: 30°24'45" N/97°43'02" W.

Barlow Hollow Spring: 30°22'20" N/ 97°46'15" W. Salamanders appear to be abundant at this locality, very close to Stillhouse Hollow Springs.

Bull Creek Spring Pool (=Bull Creek Spring #1): 30°24'59" N/97°49'00" W. This locality is not a spring, but an isolated pool left from periods of higher flow. Source spring to be determined.

Bull Creek Spring Tributary Spring (= Bull Creek Spring #2): 30°25'38" N/ 97°49'08" W.

Pedernales Spring #2 (= Hammett's Crossing Spring #2). 30°20'23" N/ 98°08'15" W, directly across the Pedernales River from Westcave Preserve. Salamanders are common in this spring and another nearby spring (see Chippindale et al. 1990). Other springs along the river in the same area remain to be investigated. Prior to our work, *Eurycea* were thought to be absent from this region (Sweet 1978, 1982); these populations appear to be geographic isolates.

Williamson Co.

Buford Hollow Spring: approximately 30°39'39" N/97°43'36" W, below Lake Georgetown dam; precise location needs to be confirmed by another site visit.

Buttercup River Cave (=Buttercup Creek Cave): near Cedar Park; precise location to be determined. Part of a major, recently discovered underground water system, inhabited by large troglobitic salamanders of uncertain taxonomic status.

Cedar Breaks Trail Spring: 30°39'36" N/97°45'02" W. *Eurycea* are abundant at this locality and nearby Crockett Garden Springs (see below). Many other springs that probably contain *Eurycea* are present along the shores of Lake Georgetown. These remain to be investigated; access to many would be easiest by boat.

Crockett Garden Spring (= Knight Spring): 30°39'50" N/97°45'04" W. Previously reported as a negative locality for *Eurycea* by Sweet (1978, 1982).

Testudo Tube (=Turtle Trot Cave, =Turtle Trap Cave, =Tortuga Cave): precise location to be confirmed. We have obtained several large *Eurycea* of uncertain taxonomic status from this cave, one of the recently discovered Cedar Park (Buttercup Creek) area caves.

APPENDIX 2. Genotype frequencies at eight enzyme-encoding loci that distinguish the San Marcos Springs population of *E. nana* from the Comal Springs population that is sometimes assigned to this species. The two populations share at least some alleles at each of the remaining 16 loci that were surveyed. These data are cumulative and include information provided in our last interim report (Chippindale et al. 1990). These data are based on 11 specimens from Comal Springs and 12 from San Marcos Springs.

X = not resolved

? = not clearly resolved, need to repeat

* = allele unique to San Marcos population (not observed in any other populations of Texas neotenic salamanders).

		<u>Genotype frequency (N individuals)</u>	
		<u>San Marcos Springs</u>	<u>Comal Springs</u>
<u>Locus</u> ¹	<u>Genotype</u> ²		
Aco-1	bb	0	4
	be	0	4
	ee	0	3
	dd	12*	0
Ak	aa	12*	0
	bb	0	11
Aat-S	aa	0	3
	ab	0	6
	bb	0	1
	ee	12*	0
	?	0	1
Gr	aa	0	10
	cc	12*	0
	X	0	1
Pep-A	bb	0	11
	gg	4*	0
	?	8	0

Appendix 2 (continued)

		<u>San Marcos Springs</u>	<u>Comal Springs</u>
Pep-B	bb	0	11
	dd	12	0
Pep-D	cc	0	11
	hh	11*	0
Pgdh	bb	0	10
	ee	12	0
	?	0	1

Notes:

1. Locus abbreviations are as follows: Aco-1= aconitate hydratase 1; Aat-S = cytosolic aspartate aminotransferase; Gr = glutathione reductase; Pep-A = dipeptidase A (glycyl-leucine); Pep-B = tripeptidase B (leucyl-glycyl-glycine); Pep-D = dipeptidase D (phenylalanyl-proline); Pgdh = phosphogluconate dehydrogenase.

2. Alleles are designated by lower-case letters (e.g. "aa" means homozygous for the "a" allele, "ab" means heterozygous for the a and b alleles).