Section 6 (Texas Traditional) Report Review

Form emailed to FWS S6 coordinator (mm/dd/yyyy): 12/6/2012

TPWD signature date on report: 10/15/2012

Project Title: Status of Newly Discovered Cave and Spring Salamanders (Eurycea) in Southern Travis and Northern Hays Counties

Final or Interim Report? Final

Grant #: TX E-122-R-1

Reviewer Station: Austin ESFO

Lead station concurs with the following comments: NA (reviewer from lead station)

Interim Report (check one):

- ☐ Acceptable (no comments)
- ☐ Needs revision prior to final report (see comments below)
- ☐ Incomplete (see comments below)

Final Report (check one):

- ☐ Acceptable (no comments)
- ☒ Needs revision (see comments below)
- ☐ Incomplete (see comments below)

Comments:

Comment for TPWD: Please correct the spelling of Dr. Chippindale's last name to "Chippindale" instead of "Chippendale"

Comments for Dr. Chippindale:

Please provide georeferences for the localities mentioned in the report (particularly for Cold Spring, Taylor Springs, Spillar Ranch, and Blowing Sink Cave).

Page 6 , 2nd paragraph – Please break the first sentence of this paragraph into two sentences, as it is very lengthy. Also correct the statement "although plan to so" to "although plan to do so"

Page 6, 3rd paragraph – Please rewrite the first sentence of this paragraph, as it is very lengthy and confusing.

Please correct the spacing issues that occur throughout the document (for example, after the phrase "…from areas outside of the main springs" on page 7 in the 3rd paragraph and in the 1st paragraph under the Results and Discussion section on page 12 at the beginning of the 5th sentence after the word "Those"

Overall, we find the research conducted to be state of the art and the report to be very well written. However, we find the taxonomic “status” of some of the populations discussed, such as those at Blowing Sink and Cold Springs, a little unclear. Is this the author’s intention or can the taxonomic assignments at each of the locations sampled be more clearly articulated within the document?

FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-122-R-1

Endangered and Threatened Species Conservation

Status of Newly Discovered Cave and Spring Salamanders (*Eurycea*) in Southern Travis and Northern Hays Counties

Prepared by:

Dr. Paul Chippendale

Carter Smith
Executive Director

Clayton Wolf
Division Director, Wildlife

15 October 2012

FINAL REPORT

STATE: ____Texas_____ **GRANT NUMBER:** ___E – 122-R-1___

GRANT TITLE: Status of Newly Discovered Cave and Spring Salamanders (*Eurycea*) in Southern Travis and Northern Hays Counties

REPORTING PERIOD: ____17 Sep 09 to 16 Sep 12

OBJECTIVE(S):

To examine the status of newly discovered salamander populations in the Barton Springs Segment of the Edwards Aquifer using mitochondrial and nuclear DNA sequences, microsatellite markers, and allozymes and assess their relationships to the federally protected species *Eurycea sosorum* and *E. nana* over two years.

Segment Objectives:

Task 1. Sept.-Dec. 2009: fieldwork will be conducted at approximately 5-6 spring sites and 1 cave site, in addition to searches for additional localities. Initiate lab work using molecular markers already developed, and explore possible additional markers.

Task 2. Jan. 2010 - Dec. 2010. Continue fieldwork, having established sampling sites and additional landowner permission. Reconstruct a phylogenetic tree and a mt haplotype network to ascertain whether the "intermediate" populations are monophyletic, and how the pattern of (primarily) mt gene flow reflects the evolutionary history of *E. sosorum*, *E. nana*, and salamanders from the newly discovered populations (and relationships with other Texas *Eurycea*). We also will assess allozyme variation.

Task 3. Fieldwork will continue. Using microsatellite loci previously developed, samples will be screened to evaluate the potential for identifying individuals by population and tracking gene flow. We will use at least 10-12 microsatellite markers per individual in all specimens collected to compare to approximately 50 *E. nana* and 100 *E. sosorum*, plus numerous representatives of other *Eurycea* in the region. Test for the presence of distinct populations and potentially identify hybrid zones.

Task 4. Jan. 2011-Aug. 2011. Complete fieldwork, molecular studies, data analyses, and prepare publication(s) for peer-reviewed publication.

Significant Deviation: None.

Summary Of Progress: Please see Attachment A.

Location: Travis and Hays County, TX

Cost: ___Costs were not available at time of this report.__

Prepared by: Craig Farquhar **Date:** 15 October 2012

Approved by: _____ **Date**: 15 October 2012
C. Craig Farquhar

ATTACHMENT A

Final Report:

Status of newly discovered cave and spring salamanders (*Eurycea*) in southern Travis and northern Hays Counties

Section 6 TX E-122-R

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Introduction and background

The Barton Springs salamander (*Eurycea sosorum*) was described by Chippindale et al. (1993) from Barton Springs in Zilker Park, Travis County, Texas. This completely aquatic species is thought to have one of the smallest geographic ranges of any amphibian in North America, and is highly susceptible to degradation of water quality in the Barton Springs segment of the Edwards Aquifer (e.g., Chippindale and Price 2005). *Eurycea sosorum* belongs to the clade *Notiomolge* (Hillis et al. 2001), separated from the *Septentriomolge* ("northern") clade by the Colorado River, an ancient feature of the Edwards Plateau that separates the Aquifer waters roughly north-south into distinct geological and biological units. A recently discovered member of the highly divergent, cave-dwelling *Typhlomolge* clade (*E. waterlooensis*) was described by Hillis et al. (2001) from subterranean waters beneath Barton Springs and appears to be partially sympatric with *E. sosorum*. The other recognized species of *Eurycea* that are geographically closest to the known range of *E. sosorum* occur along and near the southeastern margin of the Plateau (Balcones Escarpment). These are *E. nana* at San Marcos Springs, Hays Co., *E. rathbuni* in the subterranean waters of the San Marcos Pool of the Aquifer beneath the habitat of *E. nana*, *E. pterophila* in the Blanco River drainage, and the little-known species *E. robusta* in the Aquifer underlying this drainage.

Despite the presence of apparently suitable spring and cave habitat in the roughly 50 km zone between the ranges of *E. sosorum* and the other species, salamanders seemingly were absent from this area (e.g., Sweet 1982, Chippindale et al. 2000). In the early 2000s, cavers discovered *Eurycea* in Blowing Sink Cave in south Austin (see Fig. 1 for this and other localities), potentially extending the known range of *E. sosorum*. However, Hillis and colleagues (pers. comm.) found that the mitochondrial haplotype of the (then) single known salamander from this site (and that of subsequently discovered animals at Taylor Springs, Hays County, geographically intermediate between Austin and San Marcos) was most similar to that of *E. nana* from San Marcos Springs. Nathan F. Bendik (2006; unpubl. master's thesis, University of Texas at Arlington) and Chippindale conducted much more extensive genetic sampling of *E. sosorum*, *E. nana*, and the few known specimens from the newly-discovered localities and found that this haplotype (or ones that are nearly identical) also occurs at roughly 30% frequency in *E. sosorum* from Barton Springs. More recently, Bendik (now with City of Austin Watershed Protection Department), Andrew G. Gluesenkamp (TPWD) and I found these *E. nana*-like haplotypes in salamanders from Cold Springs in Austin, the third identified "new" locality (this site is roughly northwest of Barton Springs). Additional sampling from Cold Spring, Taylor Springs and Blowing Sink Cave (detailed in this report) confirms the occurrence of this haplotype group alone at these geographically proximal or intermediate localities. Very recently (August 2012), Bendik and Gluesenkamp (after years of attempts) obtained access to a spring on the Spillar Ranch, roughly halfway between the Blowing Sink and Taylor Springs sites, and obtained specimens, filling a major geographic sampling gap. Here I show that these too possess "*nana*-like" haplotypes, and that haplotypes unique to *E. sosorum* have only been observed at Barton Springs.

Occurrence of the "*nana*-like" haplotypes could represent retention of ancestral mitochondrial sequence polymorphism. However, the two species probably diverged from a common ancestor over 2 million years ago (Bonett, Wiens and Chippindale, in prep; see also Wiens et al. 2006 for divergence times in other Texas *Eurycea*). Thus, the minimal differentiation between the "*nana*" and "*nana*-like" haplotypes may indicate that there has been some degree of gene flow since speciation (likely limited and/or sporadic). This hypothesis is supported to some extent by the mitochondrial and nuclear data presented here, but it appears very unlikely that regular gene exchange between the two species is occurring. Moreover, the molecular data and analyses indicate that any past gene flow likely was unidirectional (south to north; i.e., San Marcos Pool to Barton Springs segment), consistent with flow paths in the Aquifer regions (see references in next paragraph).

Although *E. sosorum* and *E. nana* from the type localities (Barton and San Marcos Springs, respectively) are distinguishable based on morphology from one another and from other central Texas *Eurycea* (e.g., Chippindale 1998, 2000; Chippindale et al. 2000, although see Results and Discussion regarding the existence of occasional individuals at Barton Springs that show characteristics of both *E. sosorum* and *E. waterlooensis*), salamanders from the geographically intermediate populations and Cold Springs are morphologically quite variable and, based on cursory examination, show external features associated with both species (I have not conducted detailed morphological/morphometric studies, although plan to so as part of a collaborative project with Bendik, Gluesenkamp, and J. Meik and C. Roelke from UTA). This further supports the possibility that *E. nana* and *E. sosorum* have hybridized to some extent although again, the molecular data and analyses argue against ongoing gene flow. While the Barton Springs and San Marcos portions of the Aquifer often are considered hydrologically separate, there is geologic evidence of periodic water flow between the two regions (e.g., Slade et al. 1986; see also the dye trace flow paths of Hauwert et al. [2004] in Fig. 1, and a very recent, much more extensive assessment provided by Johnson et al. 2012). Moreover, dye traces show that water from the Blanco River can emerge at either spring (Johnson et al. 2012).

An initial hypothesis in this study was that salamanders from at least some of these sites could represent a third, closely related species, and Taylor Springs (the site most distant from Barton Springs) in particular appears to be highly isolated from gene flow with the other populations and species addressed here, although as shown below its allelic composition with respect to the nuclear sequences and microsatellite loci is most consistent with that of *E. sosorum*; it simply lacks most of the variation seen in the Barton Springs, Cold Spring, Blowing Sink Cave, and Spillar Ranch Spring populations. Further assessment of the status of this population is provided in the Results and Discussion below. Conversely, analyses of microsatellites and nuclear sequences suggest relatively high levels of gene flow between salamanders from Barton Springs, Cold Spring and Blowing Sink (the sites closest to Barton Springs) and Spillar Ranch Spring (next closest) and these almost certainly represent *E. sosorum*. Although the distribution of salamanders in northern Hays and southern Travis Counties is little known, the sampling in this study provides a transect with few, but well-spaced, localities, and the

inferences as to their status and levels of gene flow largely follow the distributional pattern.

Although not initially intended as a major focus of this study, the discovery of mitochondrial haplotypes otherwise associated with *E. waterlooensis* in two individuals from Barton Springs identified based on morphology as *E. sosorum*, and a mitochondrial haplotype associated with *E. sosorum* in an individual identified as *E. waterlooensis*, led me to add this species to the study. One of the key questions initially was whether *E. sosorum* and *E. nana* hybridize, and inclusion of *E. waterlooensis* was necessary to further test the distinctiveness of *E. sosorum*. As I explain in more detail below, mitochondrial, nuclear sequence, and microsatellite data indicate that gene exchange between *E. sosorum* and *E. waterlooensis* is very limited and the two maintain distinct identities while effectively sympatric. Limited comparisons with *E. rathbuni* reveal an intriguing situation: like *E. sosorum*, *E. waterlooensis* contains two distinct mitochondrial haplotype clades, one very similar to a haplotype clade seen in *E. rathbuni* from San Marcos, suggesting a parallel between events that may have shaped mitochondrial variation in *E. sosorum* and those that have done so in *E. waterlooensis*.

Chippindale et al. (2000) found that *E. nana* from San Marcos Springs (the type locality and only confirmed habitat for this species) were extremely distinct from all other Texas *Eurycea* based on multiple allozyme loci. *Eurycea sosorum* from Barton Springs displayed none of *E. nana*'s diagnostic (nuclear) allozyme alleles, but did exhibit the highest levels of allozyme-based genetic variation seen in the Texas *Eurycea*. This suggests large population size and/or input of genes from areas outside of the main springs . However, at the time, additional populations were not known, nor was the existence of *E. waterlooensis* (in the subterranean waters beneath Barton Springs), so gene flow seemed unlikely. Given recent discoveries, this now seems much more plausible, not only from populations between San Marcos and Austin, but likely between *E. sosorum* and *E. waterlooensis* (to a limited but detectable extent; see below). The apparent lack in *E. sosorum* from Barton Springs of the unique allozyme alleles seen in *E. nana* from San Marcos Springs suggests that direct gene flow from San Marcos to Austin is unlikely.

Collaborators:

This work has been conducted in close conjunction with Nathan Bendik (City of Austin Watershed Protection Department) and Andy Gluesenkamp (Texas Parks and Wildlife Department), and when I say "I" in this report it is important to recognize that although I conducted the molecular work and am responsible for the conclusions, they have devoted a tremendous amount of field time, as well as providing highly valuable insights. They have greatly increased the sampling of the three recently discovered populations, Cold Spring, Blowing Sink, and Taylor Springs (plus Spillar Ranch Spring, which was thought to be inhabited by salamanders but only became accessible in August 2012, after a hiatus of over 20 years). In the course of this work, they identified multiple additional sites that have high potential for occurrence of *Eurycea*. Gluesenkamp and Bendik have spent an estimated 120 person-hours in the field, including repeated sampling of six test wells that

intersect the subterranean waters through which these salamanders move, plus four springs with potential for salamander occupation in Travis County. Although salamanders have not yet been found in the wells, the work did yield important information on subterranean invertebrates, and access to Spillar Ranch Spring filled a major geographic gap in sampling.

Methods

Sampling:

Salamanders, or tissue samples (tail tips) from animals that subsequently were released or maintained in the City of Austin breeding facility, were collected from the known ranges of the species involved (*E. sosorum*, *E. nana*, and *E. waterlooensis*), plus the "new" localities described below (Fig. 1). Most samples from the "new" populations were obtained in 2009-2012 (primarily by Gluesenkamp and Bendik), but some from the earlier-known localities represent material that colleagues and I collected in the early to mid-1990s. DNA samples for numerous specimens of *E. nana*, collected by Lauren Lucas (former graduate student, Texas State University), were provided by Dr. Christopher Nice (Texas State University). In additional, many samples of *E. sosorum* and *E. waterlooensis* were provided by Dee Ann Chamberlain and Dr. Laurie Dries (City of Austin Watershed Protection Department), many in the course of a study that I conducted with them several years ago on genetics of *E. sosorum* at Barton Springs. All recently collected specimens were or will be deposited in the University of Texas at Arlington Amphibian and Reptile Diversity Research Center collection, and earlier specimens are held in the Texas Natural History Collection at the University of Texas at Austin. Georeferences for all localities are available from the City of Austin Watershed Protection Department and the Texas Parks and Wildlife Department.

This study involves a much larger sample than was previously available (five individuals from Cold Spring (Austin, geographically closest to Barton Springs), 16 from Blowing Sink (south Austin), eight from Spillar Ranch Spring, (extreme south Austin, previously unsampled), and 10 from Taylor Springs (Little Bear Creek; Onion Creek drainage), in addition to dozens of individuals of *E. sosorum* and *E. nana* from their type localities at Barton Springs and San Marcos Springs, respectively (for microsatellites, 69 from Barton Springs and 43 from San Marcos Springs). The original questions in this study focused on possible interactions between *E. sosorum* and *E. nana* (whether mitochondrial genes from *E. nana* were introgressing into *E. sosorum*, and if the two may even be conspecific). However, it became evident (or at least, it appears very likely) that *E. waterlooensis* hybridizes with *E. sosorum*, so I included representatives of this species in the study (up to 16 depending on the genetic locus examined). I have considerable data for *E. rathbuni* at San Marcos Springs (this subterranean species is thought to be sister or very closely related to *E. waterlooensis*) and have used this to a limited extent for reference, but there is no evidence that it hybridizes with *E. nana*, with which it is effectively sympatric. Thus, it was not included directly in the study except for comparison to *E. waterlooensis*, primarily with respect to mitochondrial DNA data.

DNA sequence markers:

DNA from most specimens was extracted using Qiagen DNeasy kits; samples from the 1990s were obtained using standard phenol-chloroform methods. Loci (here I use this term to mean any specific segment of DNA, mitochondrial [mt] or nuclear [nuc], coding or non-coding) were amplified via polymerase chain reaction (PCR) using a wide range of cycling conditions and in most cases, taxon-specific primers developed or modified by me and students in my laboratory. I sequenced portions of a broad range of mt and nuc loci. Mt: cytochrome b (approximately 1.1 kilobase [kb], trimmed to 979 base pairs [bp]) was the primary marker used, but cytochrome oxidase I (COX1; approx. 700 base pairs, bp), NADH dehydrogenase subunit 2 (ND2) and adjacent tRNA genes (approx. 900 bp), and other mt regions were used in some cases, partly to verify the occurrence of apparent hybrids between *E. sosorum* and *E. waterlooensis*. Nuc: Portions of recombination-activating gene I (RAG-1; approx. 2 kb; I subsequently focused on a variable region of about 800 bp), melanocortin receptor gene 1 (Mc1r; approx. 500 bp), pro-opiomelanocortin gene (POMC; approx. 450 bp), triosephosphate isomerase gene from end of exon 2 through exon 5 (including three introns; approx. 800 bp), and a segment of the ornithine decarboxylase (ODC) gene encompassing mid-exon 6 to mid-exon 8, including introns 6 and 7 (approx. 650 bp). I eventually abandoned ODC because although it shows useful variation, this primarily is in the form of multiple insertion-deletion events in the introns. This becomes complex, but essentially, the sequence is "shifted" in heterozygotes and interpretation becomes extremely difficult without time-consuming and expensive cloning approaches (which proved intractable). TPI showed little useful variation and amplification was often difficult. RAG-1, POMC, and Mc1r were much more readily amplifiable and interpretable, and I focused on these for the nuc sequence component of the study.

Of the mt loci, cytochrome b (cyt b) was most variable overall, and cytochrome oxidase I and NADH 2 also were highly informative (but used to a very limited extent, and data are not shown here). Since these genes are mitochondrial they are tightly linked genetically; however, each provides a "check" on the other with respect to consistency of results. Where applied, these additional mt regions provided results consistent with those based on cytochrome b.

Of the nuc regions sequenced, all showed little variation (each is known to be evolutionarily conserved, so this was to be expected; less is known for the the introns of ODC, and this locus largely has been used phylogenetically for studies of relationships in birds [e.g., Johansson et al. 2008]). However, the sites that are variable (in RAG-1, POMC, and Mc1r) have proven to be informative (Table 1), especially with respect to the possibility of hybridization. Many workers avoid such slowly evolving genes for study of populations and closely related species. However, the changes that do occur are likely only to have occurred once (i.e., the sites are unlikely to change back and forth between, say an A and a G). Thus, they can serve as highly reliable markers of organismal history on even a fine scale even when the vast majority of sites in the gene are invariant.

RAG 1 exhibited five potentially informative variable sites across *E. nana*, *E. sosorum*, the populations in question, and *E. waterlooensis*. It is very important to note that each site within the genes cannot be treated as an independent locus since they are so closely genetically linked; an allele of the gene is defined by the particular combination of variation at the sites. Standard sequencing methods often cannot separate individual alleles, and an allelic library that I developed using cloning approaches proved problematic, although this approach is worth revisiting, especially for fine-scale studies of parentage and relationships among individuals. As I describe below, sequencing can define alleles that are mutually exclusive, and here this is the case for RAG-1 and Mc1r. For Mc1r, two sites were variable in the taxa of interest, and three (consisting of a covarying nucleotide "doublet (i.e., two adjacent sites that consistently varied together) together with a third elsewhere in the sequence region examined, were informative for POMC.

Microsatellites:

The microsatellite portion of this study initially was hampered by technical problems. I have conducted extensive microsatellite studies of the Texas *Eurycea* and this was very unexpected. Recently I was able to get these markers working reliably and have made a major push to complete this data set and verify results. This has been highly successful, but there are some missing data to fill in and data points to be checked. At this stage, the small amount of remaining work to be done seems highly unlikely to affect the overall results, and I can add this information to the final version of the report during the review period. The problems largely revolve around *E. nana*, somewhat surprising given the high quality of the DNA and its fairly close relationship to *E. sosorum* and the populations of uncertain status.

PCR primers for microsatellite loci were developed using a modification of the methods of Glenn and Schable (2006), and PCR products were electrophoresed on an ABI 3130xl automated sequencer, generally multiplexed with combinations of HEX, FAM, and NED dyes and sized using ABI's 400 or 500 bp ROX size standards. Loci used are listed in Table 5 together with raw allelic data. Data for most individuals of *E. sosorum* (Barton Springs) were collected in an overlapping study with Austin Community Foundation (Chippindale 2011), and many were retested here and used for calibration of mobilities. Of roughly 60+ loci tested (most prior to this study) I initially narrowed this to eight. Three of these proved erratic or difficult to interpret (although I have used them previously in other species of Texas *Eurycea*, including *E. sosorum*); the main problematic taxon was *E. nana*, for which amplification of three loci was sporadic, despite the availability of dozens of high quality DNA samples. This limits the results to use of relatively few, somewhat conserved loci (although they did exhibit up to 24 alleles/locus, not unusual for a microsatellite study), and as a result the levels of divergence may be underestimated in comparison to those of studies that use more variable markers. However, particularly with use of R_{st}-based estimates of divergence as well as F_{st}s (the former take into account not only frequencies of individual alleles, but differences in allele size, a function of repeat number; see below) insights into levels and

patterns of differentiation are enhanced. The loci used do not introduce a systematic bias in terms of relationships or gene exchange; they simply represent a conservative estimate of the level of genetic isolation; and the difficulties with *E. nana* are suggestive of its distinctiveness, consistent with previous use of nuclear allozyme loci (Chippindale et al. 2000).

The loci used all vary primarily based on differences in numbers of tetranucleotide repeats. To ensure that I was examining genuine microsatellites (especially because some PCR primer pairs generate multiple peaks of widely varying sizes), I gel-extracted and sequenced bands from agarose gels to verify the identity of these markers.

Overall, I employed a wide range of markers that reflect genetic variation across both nuclear and mitochondrial genomes, and directly answer questions about potential hybridization between *E. nana* and *E. sosorum*, as well as possible gene flow between *E. sosorum* and *E. waterlooensis*.

Analyses:

Results of phylogenetic analyses shown here are based on the mt cytochrome b gene. In the tree presented (Figure 2) I did not include all specimens of *E. sosorum* (Barton Springs) and *E. nana* (San Marcos) because the additional specimens examined exhibited sequences that identical or nearly identical to those in their haplotype groups. This is a matter of simplicity (the tree becomes difficult to read if all samples are included), and these can easily be added if this is desirable. I present a neighbor-joining tree based on HKY85-corrected sequence distances (Hasegawa et al. 1985); Bayesian analysis was also employed and yielded nearly identical results. Standard methods of phylogenetic analysis are problematic for the conserved nuclear genes because there are very few variable sites, and many individuals are heterozygous for some of these sites. However, the information they contain provides a great deal of insight into gene flow and here is presented in tabular form.

Allelic designations for microsatellites, defined by relative fragment size, were determined using GeneMarker (Softgenetics), and population genetic analyses primarily were conducted using GenAlex 6 (Peakall and Smouse 2006). Loci were treated as codominant markers, and results of analyses (with respect to population differentiation and potential gene flow) focus mainly on Wright's F statistics (e.g., Wright 1969; see also Weir and Cockerham 1984), plus the R- statistics of Slatkin (1995; see also Slatkin 1981, 1985). F-statistics simply consider each allele for a given locus as distinct, regardless of repeat number or fragment size, whereas R values take into account the difference in fragment size based on a stepwise model of microsatellite evolution, whereby increasing difference in size reflects increasing time since divergence. I used AMOVA (Analysis of Molecular Variance; Excoffier et. al. 1992) to determine the extent to which variation in microsatellites is partitioned among populations (and species) relative to total variation. I included the highly distinct subterranean species *E.*

waterlooensis, which is effectively sympatric with *E. sosorum* at Barton Springs (Hillis et al. 2001; Chippindale et. al. 2000, Chippindale 2000) because as described further below, it seems very likely that the two experience some degree of hybridization.

Results and Discussion

Mitochondrial variation:

With the exception of *E. sosorum* and *E. waterlooensis* at Barton Springs, salamanders at each locality exhibited little or no mt variation based on cytochrome b sequences (Fig. 2). Within *E. nana* from the type locality, San Marcos Springs (13 specimens included in the tree), uncorrected sequence divergence ranged from 0.0 - 0.2% and these form a weakly supported monophyletic group. They fall within the larger clade of *E. nana*-like sequences seen at Cold Spring, Blowing Sink, Spillar Ranch Spring, Taylor Springs, and a subset of individuals from Barton Springs. All mt divergences in this haplotype group are low (maximum about 0.3%). Those from Spillar Ranch Spring (more distant from Barton Springs; five with complete sequence) exhibited no mt variation, nor did those from Taylor Springs (geographically closest to San Marcos Springs; 10 individuals).

The situation at Barton Springs is complex. There are two distinct mt haplotype clades among specimens identified based on morphology as *E. sosorum*. Within each haplotype clade almost all individuals exhibit identical or near-identical cyt b sequences. One constitutes the *E. nana*-like group described above (approximately one third of those examined from Barton Springs plus all of those from the localities of uncertain status; some of these not shown in the tree are represented by incomplete sequences but all are assignable to haplotype clade). The other is a distinct group differentiated by up to about 2.5% from the *E. nana*-like haplotype clade. The latter are sister to a relatively geographically proximal ("southeastern", i.e. *Blepsimolge* clade sensu Hillis et al. 2001) but well-differentiated group (about 3.0%) consisting of *E. latitans*, *E. pterophila*, and *E. neotenes*. The type specimen of *E. sosorum* is a member of this clade (Chippindale et al. 1993; Chippindale et al. 2000), and the existence of the *E. nana*-like mt clade was only discovered later. It is important to note that *E. sosorum* was described based not only on mt differentiation, but also nuclear (allozyme) markers and morphology.

To (seemingly) complicate the mt results further, one of 14 individuals of *E. waterlooensis* for which complete or near-complete cyt b sequences are available falls within the second of the mt clades of *E. sosorum* described above (although it exhibits approximately 1.0% divergence from almost all others in the clade), and two *E. sosorum* appear (mitochondrially) most closely related to *E. waterlooensis*. In turn, *E. waterlooensis* is divided into two mt clades, one distinct among Texas *Eurycea*, and the other very similar to a subset of *E. rathbuni* (known from subterranean waters beneath San Marcos Springs, ranging south to the New Braunfels region [Chippindale et al. 2000; Chippindale 2008; and references therein]). The former haplotype clade in *E. waterlooensis* is sister to, but about 2.0% divergent from, another haplotype clade within *E. rathbuni*.

Mitochondrially, the situation appears confusing, to say the least. But again, both *E. sosorum* and *E. waterlooensis* are morphologically distinct from other Texas *Eurycea* (and usually from one another), and mtDNA does not tell the whole story.

Nuclear sequence variation:

Details of gene-by-gene and site-by-site variation are given in Table 1. As explained earlier, each site within a gene cannot be treated as evolving completely independently of the other sites (because of the tight genetic linkage) and specific alleles (i.e., chromosome-specific sequences for each gene) have not been verified in many cases. I use the terms homozygous and heterozygous to refer to specific bases at particular sites within the gene, but this should not be construed to mean that each is a distinct allele (although if sequence were only available for the region containing a variable site, the single site could define an allele). However, the patterns of variation are highly informative, and in some cases, full alleles that appear to be unique to *E. nana* or *E. waterlooensis* are identifiable. For instance, all 24 individuals of *E. nana* from San Marcos Springs are homozygous for T at RAG-1 site 4, a condition not seen in specimens examined from any other locality (thus they unquestionably possess and appear to fixed for a unique allele of this conserved gene). This is indicative of genetic isolation of this taxon. *Eurycea waterlooensis* in particular exhibits high frequencies of particular bases not seen in most other populations/taxa, although in many cases there is overlap (with substantial differences in frequency, presumably reflecting patterns of allelic variation) with the sympatric taxon *E. sosorum* at Barton Springs and in many cases the populations at Cold Spring and Blowing Sink, geographically closest to Barton Springs.

Specimens from the two localities most distant from both Barton and San Marcos Springs (Spillar Ranch Spring and Taylor Springs) display no intrapopulation nuclear sequence variation and possess sequences (and alleles) for POMC and Mc1r seen in specimens from Barton Springs, but lack the RAG-1 allele specific to *E. nana* (San Marcos Springs) and the Mc1r allele seen in *E. nana* and some individuals from Barton Springs and Blowing Sink. The low levels or lack of variation in mitochondrial and nuclear sequences (and microsatellites for Taylor Springs; see below) suggests that Taylor Springs in particular is a small, isolated population, not part of a larger continuum of ongoing genetic exchange, whereas the status of gene flow for Spillar is less clear. Almost all variation in the nuclear sequences occurs at Barton Springs, Cold Spring, and Blowing Sink (Table 1), and in many cases coincides with that seen in *E. waterlooensis* (i.e., at variable nuclear sites, most individuals at these localities, pooled, are homozygous for an "*E. sosorum* base", a small fraction are homozygous for the "*E. waterlooensis* base", and a larger proportion are heterozygous for the "*E. waterlooensis* base" and the "*E. sosorum* base"). This variation also suggests that there is considerable genetic exchange among the populations at Barton Springs, Cold Spring, and Blowing Sink (but see discussion of the microsatellite results).

With respect to the three individuals at Barton Springs that exhibit mt haplotypes inconsistent with their identification based on morphology (two *E. sosorum* of 70+ with

haplotypes extremely similar to those of the Barton Springs-specific *E. waterlooensis* mt clade, and one *E. waterlooensis* of 15 with a haplotype similar to that of the main, Barton Springs-specific *E. sosorum* haplotype group), none show nuclear sequence variation consistent with the mitochondrial haplotype of the other species, nor do they appear, across nuclear genes including microsatellite loci, to be F1 hybrids (Tables 1 and 5). This is consistent with a pattern of occasional hybridization, limited mt exchange, and likely some introgression of nuclear genes. It is not at all consistent with high levels of gene flow, and *E. sosorum* and *E. waterlooensis* clearly maintain their identities as distinct lineages despite their occurrence at the same locality (*E. waterlooensis* has only been found at Barton Springs, but surveys at Cold Spring and Blowing Sink have been extremely limited).

Microsatellite variation:

Summary statistics regarding heterozygosity for microsatellite loci are given in Table 2. Generally, levels of variation based on heterozygosity are high, as is typical for microsatellites, but most strikingly, no variation is seen for three of the five loci in Taylor Springs, further emphasizing the likelihood that it represents a small, isolated population, not part of a major path of continuous gene flow. The alleles that it possesses are seen in *E. sosorum* and some of the other populations, but for two loci (A4v2 and u6), not in *E. nana* despite the fact that this population (of those whose status is in question) is geographically closest to the range of that species.

Table 3 shows pairwise F_{st} and R_{st} values for the populations and species based on the combined microsatellite data and estimated Nm (number of migrants per generation; see Slatkin 1981, 1985, Neigel 2002). F_{ST} and R_{ST} can vary between 0 (completely free interbreeding among individuals in a geographic context) and 1 (completely different allelic composition for the markers studied, reflecting near-unambiguous isolation from gene flow). Although such measures are not necessarily directly comparable across studies (or different kinds of data), as a very rough rule of thumb, values of F_{ST} from 0.0-0.05 indicate low levels of genetic fragmentation (and thus high levels of gene flow, expressed as Nm, the number of migrants per generation); from 0.05-0.15 moderate differentiation; from 0.15-0.25 high differentiation, and from 0.25 - 1.0 very strong differentiation (e.g., Slatkin 1985, Kindt et al. 2009).

Under either measure, estimates of gene flow are highest for pairwise comparisons among Barton Springs, Blowing Sink, and Cold Spring, and to a lesser extent Spillar Ranch Spring, although not all are reciprocal (specifically, a pairing of Barton Springs with any of the others populations of uncertain status yields a high estimate of gene flow, but pairing these other populations with one another does not necessarily give such a result). One interpretation, consistent with some hydrogeologic data (e.g., Johnson et al. 2012) is that flow of both water and genes is primarily toward Barton Springs. This may also explain why the *E. nana*-like mt haplotype is seen in all of the populations of uncertain status, and at Barton Springs, but the Barton Springs (*E. sosorum*)-specific haplotype is seen only at Barton Springs.

Surprisingly, based on F_{ST} alone, estimates of gene flow are within the range consistent with population continuity for comparisons between *E. sosorum* and both *E. nana* and *E. waterlooensis* under the interpretation that even one migrant per generation is sufficient (e.g., Slatkin 1985). It is important to note that F_{ST}'s may also reflect past events (i.e., earlier gene flow and possibly shared ancestral polymorphisms), and for plethodontid salamanders in particular, Larson (1984) argued for caution in their interpretation as indicative of solely events happening in the present.

The picture changes considerably when R_{ST} values (Slatkin 1995) are used, which many would argue are most appropriate for microsatellite data. As explained briefly above, these measures take into account not only frequencies of alleles, but their relative sizes, under a stepwise model of microsatellite evolution in which repeat units are gained and lost successively; thus the greater the difference in allele sizes, the more time has elapsed since separation and the less likely it is that gene flow is ongoing. Using this approach (Table 3), the only values and corresponding Nm's consistent with substantial gene flow are those between *E. sosorum* and Blowing Sink, Cold Spring, and Spillar Ranch Spring, the geographically most proximal sites. *Eurycea nana* and *E. waterlooensis* appear to be highly isolated (as does Taylor Springs) and very unlikely to be exchanging genes with any others at a substantial frequency.

R_{ST}'s also paint a very different picture of the partitioning of genetic variance among versus within populations. Using AMOVA (Analysis of Molecular Variance; Excoffier et al. 2001), F_{ST}'s (Table 4) indicate that overall, 73% of the microsatellite variation occurs within populations/species and 27% among. R_{ST}'s suggest a much higher degree of partitioning among units (64% among and 36% among). Thus, the latter measure suggests that considering the entire assemblage, there is a high degree of fragmentation, inconsistent with widespread gene flow.

Allozymes:

Although not examined here, allozymes (nuclear protein markers) strongly distinguished *E. nana* from San Marcos Springs (which has several unique and apparently fixed alleles at different loci) from *E. sosorum* at Barton Springs (and from all other populations and species known at the time; Chippindale et al. 2000). This also argues against direct or regular gene exchange between the two species. It would be very desirable to examine allozyme loci for the populations of uncertain status, and now there is the possibility to sequence the genes for some of the loci, based on nuclear cDNA libraries developed by my former student and colleague Ron Bonett at University of Tulsa. This exciting, and could well shed further light on the situation.

Genetic introgression revisited:

Chippindale et al. (1993) showed that, at least for the samples included in their morphometric analysis, *E. sosorum* was distinguishable from other surface-dwelling species of "southeastern" surface-dwelling central Texa *Eurycea*. This appeared to be related in part to some degree of "troglobitic" (cave-associated) morphology (e.g.,

reduced eyes). This could simply be the result of an existence that includes use of subterranean and surface habitat, but given that some degree of genetic interchange probably occurs it might also reflect the influence of some input of nuclear genes from *E. waterlooensis*. Dee Ann Chamberlain, who conducts surveys of these salamanders and maintains the captive breeding colony for the City of Austin, reports (personal communication) the occurrence of a small proportion of individuals that (subjectively; i.e., no detailed analysis has been undertaken) appear "intermediate" morphologically between *E. sosorum* and *E. waterlooensis*. We plan more comprehensive molecular analysis of these to assess whether they could be F1 hybrids. Of the three individuals from Barton Springs in this study that show mt haplotypes associated with the other species (two *E. sosorum* with *E. waterlooensis* mt haplotypes, and one *E. waterlooensis* with an *E. sosorum*-like haplotype), one of the *E. sosorum* falls into the morphologically intermediate category but appears more *E. sosorum*-like, one looks like a normal *E. sosorum*, and the *E. waterlooensis* with an *E. sosorum*-like haplotype appears based on her informal assessment to be a normal *E. waterlooensis*. Nuclear data also place them within the "correct" species based on morphology. Obviously the sample sizes are small, but these do not appear to be F1 hybrids, further suggesting that occurrence of "reversed" haplotypes may be the consequence of occasional hybridization, and that these haplotypes have stayed within the populations of each species at low frequencies; there is no evidence of mt homogenization.

Finally, it is important to note that for species that diverged relatively recently, mtDNA (which generally is thought to be non-recombining and has a small effective population size due to its haploid nature and female-only transmission), retention of ancestral polymorphisms and incomplete lineage sorting often leads to mt non-monophyly (e.g., see Wiens and Penkrot 2002, McGuire et al. 2007). Thus, with respect to the situation with *E. nana* and *E. sosorum*, we may be seeing such an effect, perhaps coupled with occasional introgression (McGuire et al. describe such situations in lizards, for example). Such introgression likely would be indirect, via the geographically intermediate populations.

Major conclusions

1) Patterns of mitochondrial variation superficially suggest the potential for a "hybrid complex" in the *E. sosorum*/*E. nana* group, perhaps involving the distantly related species *E. waterlooensis* and even *E. rathbuni*. However, this is not supported by the nuclear sequence and microsatellite evidence, which (like earlier nuclear allozyme data) distinguish *E. sosorum* at Barton Springs from *E. nana* at San Marcos Springs. The *E. nana*-like mitochondrial sequences present in a subset of the Barton Springs population and all peripheral and geographically intermediate populations are not identical to those of *E. nana* at San Marcos Springs, suggesting that if gene flow has occurred, it likely is sporadic.

2) With respect to interactions between *E. sosorum* and *E. waterlooensis*, these taxa are highly distinct morphologically (although occasional individuals that appear

"intermediate" do occur). The *Typhlomolge* clade that includes these species appears to have diverged from the *Blepsimolge* clade that includes *E. nana* and *E. sosorum* over 10 million years ago. Levels of mitochondrial exchange between *E. waterlooensis* and *E. sosorum* appear very low, and although considerable nuclear variation is shared, frequencies of alleles and sequence substitutions at multiple loci differ greatly. It is hardly surprising that two species which are effectively sympatric would hybridize occasionally; the fact that they maintain distinct identities despite regular contact is extremely strong evidence of their status as separate evolutionary lineages. Speculatively, limited genetic exchange may even be beneficial: *E. sosorum* displays very high levels of nuclear variation (often viewed as an indicator of "genetic health"), some likely derived from *E. waterlooensis*, and a slightly "troglobitic" morphology that conceivably could even enhance use of subterranean as well as surface habitat.

3) The populations of *Eurycea* at Blowing Sink and Cold Spring (and Spillar Ranch and Taylor Springs) are indistinguishable mitochondrially from the "*nana*-like" haplotype group at Barton Springs. They share nuclear sequence substitutions or alleles, and microsatellite alleles, with *E. sosorum* that are rare or undetected in *E. nana*. Although there is evidence that this group -- Barton Spring, Cold Spring, and Blowing Sink -- may not be completely panmictic (randomly interbreeding), they appear to represent the same species, and population genetic analyses of the microsatellite data reveal substantial to very high levels of gene flow, especially between Barton Springs and both Cold Spring and Blowing Sink (although gene flow appears to be toward Barton Springs, in that the *E. sosorum* - specific mitochondrial haplotype was not seen in these populations). Spillar Ranch exhibits less nuclear variation overall, but its nuclear genetic composition is consistent with that of *E. sosorum* and not *E. nana*. The Taylor Springs population is somewhat problematic; it appears to be small and isolated, with a very low level of genetic variation and little evidence of ongoing genetic exchange with any of the other populations or species. However, its genetic composition is also most consistent with that of *E. sosorum* and the other populations, exclusive of *E. nana*.

4) *Eurycea waterlooensis* contains two distinct mitochondrial haplotype clades, one different from that in other Texas *Eurycea* (with the exception two *E. sosorum* that share this haplotype, presumably through introgression), and one very similar to that seen in some *E. rathbuni* from the San Marcos area. The pattern is very similar to that observed in *E. sosorum*, i.e., one localized haplotype group and one very similar but not identical to that from a taxon (*E. nana*) associated with the San Marcos Pool of the Aquifer. Perhaps both *E. rathbuni* and *E. nana* have occasionally contributed genes to their closest relatives; for the *E. rathbuni*/*E. waterlooensis* situation, it will be very important to look more closely at nuclear data for both species, for which detailed comparisons were not made here. Possibly too, these are cases of shared ancestral polymorphism that have involved some level of fragmentation and reconnection. Based on morphology, *E. waterlooensis* appears very distinctive regardless of haplotype, and members of both haplotype groups (plus the single individual with an *E. sosorum*-like haplotype) are relatively homogeneous with respect to both nuclear sequence substitutions and microsatellites and the respective species to which they were assigned based on morphology.

5) The patterns seen here do not support the view that there is ongoing, high-level hybridization between *E. nana* and *E. sosorum*, *E. sosorum* and *E. waterlooensis*, or *E. waterlooensis* and *E. rathbuni*. If that were the case, we would expect to see homogenization of haplotype and allele frequencies across localities and species; instead, there are distinct patterns of species, population, and regional variation across a wide range of molecular markers. But there is a "signature" of past and perhaps sporadic gene flow with an apparent directionality from south to north, overlain with occasional hybridization between species at Barton Springs. Levels of genetic variation are especially high in *E. sosorum*, and it appears that salamanders at this locality may accumulate genetic variation that flows into Barton Springs as well as from *E. waterlooensis*. This in itself represents a striking case of "evolutionary potential", and highlights the need for protection of the salamanders and the complex aquatic system that they inhabit.

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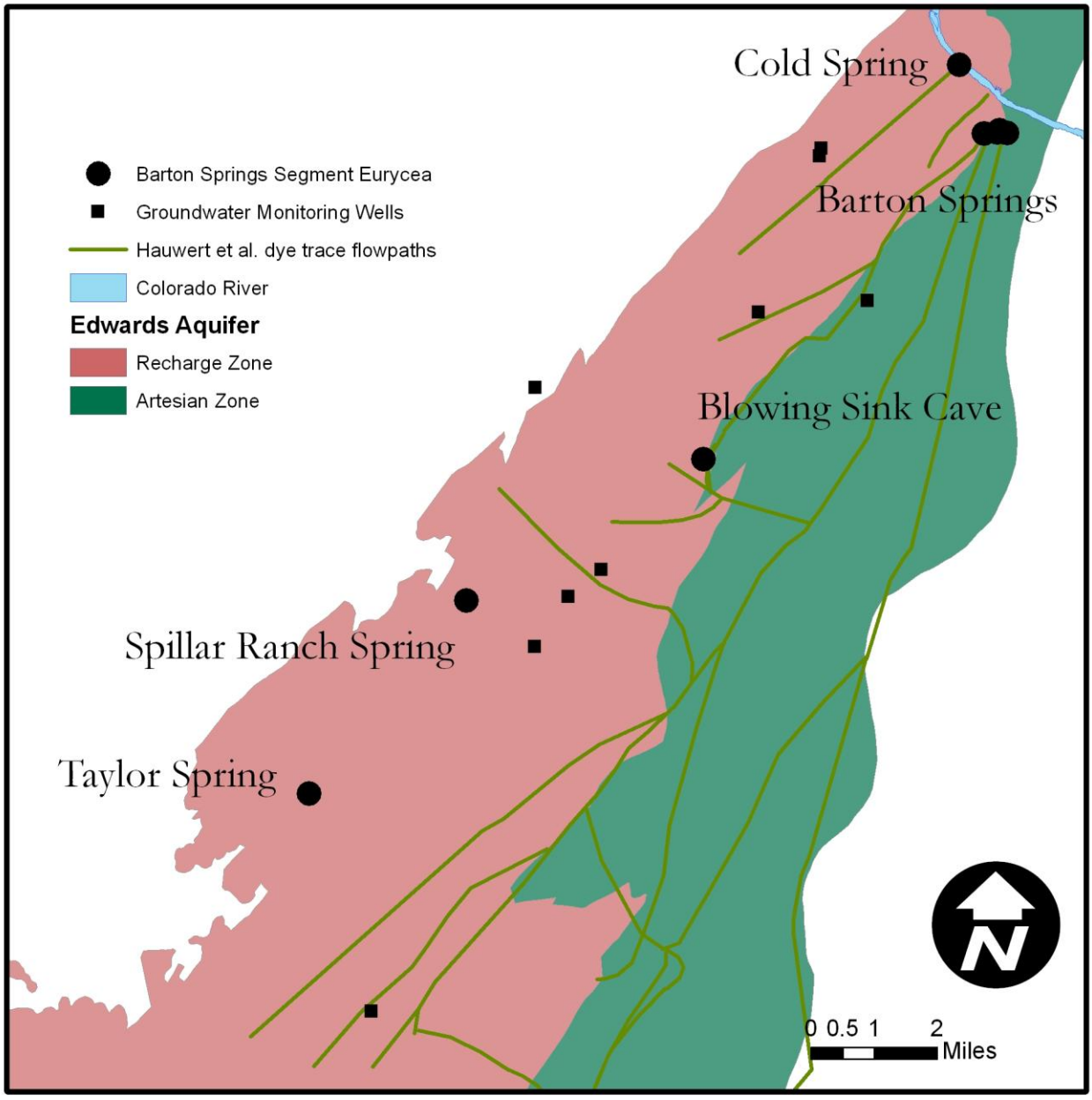

Figure 1. Primary survey and sampling region for populations of *Eurycea* of unknown status in northern Hays and southern Travis Counties. Barton Springs is the type (and thus far only confirmed) locality for *E. sosorum*; San Marcos Springs (located approximately 25 km south-southwest of the lower margin of this map) is the type and only known locality for *E. nana*. Salamanders of uncertain taxonomic status have been collected from Cold Springs, Blowing Sink, Spillar Ranch Spring, and Taylor Springs. Other potential localities in the area have been and are under investigation, including the test wells shown here.

Figure 1. Mitochondrial haplotype tree based on 979 base-pairs of the cytochrome b gene, constructed using neighbor-joining and HKY85-corrected distances. Numbers at nodes represent bootstrap values from 2000 pseudoreplicates. Only representative individuals of *E. nana* and *E. sosorum* are shown for clarity.

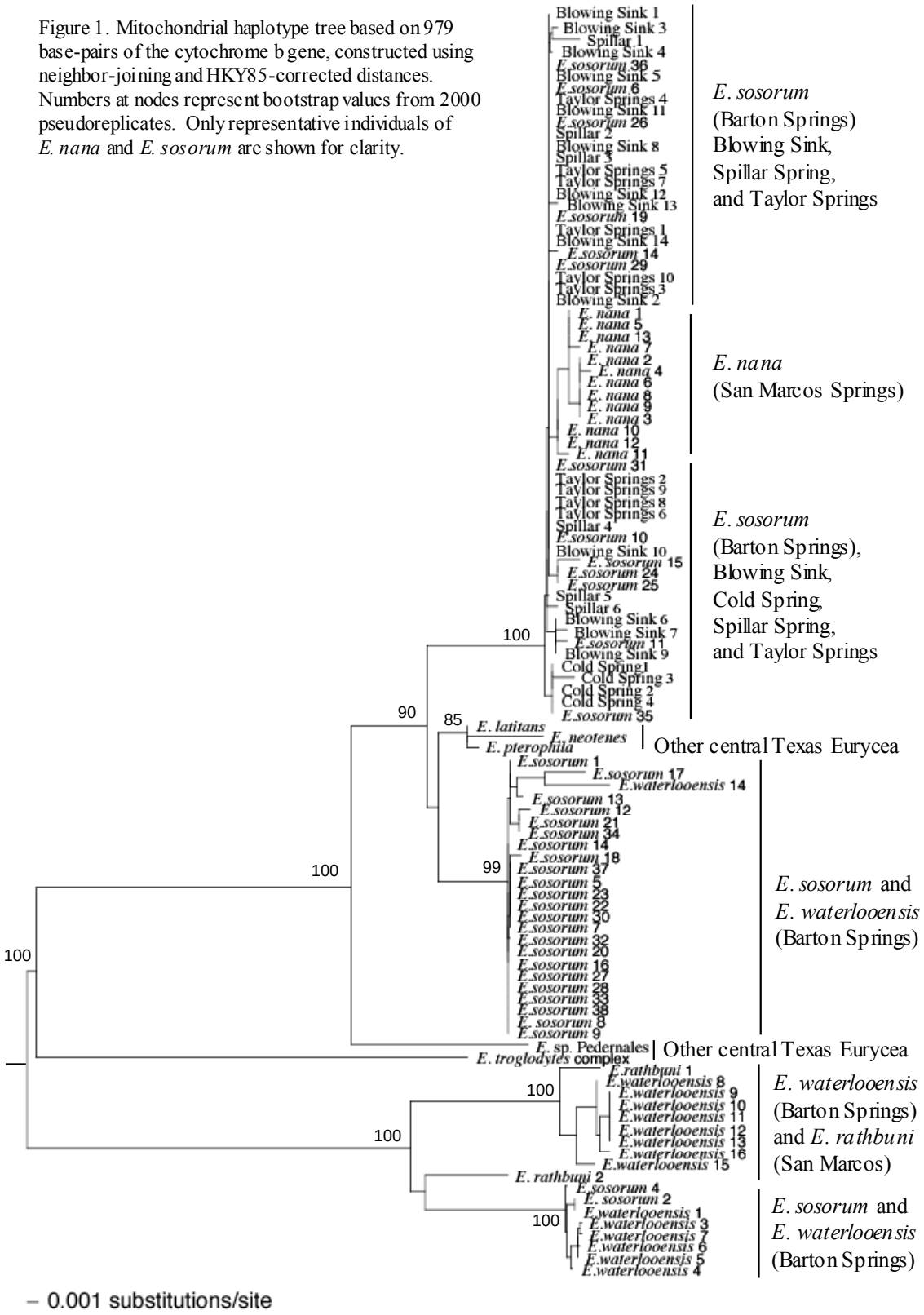

Table 1. Site-specific variation in nucleotide composition across species and populations for portions of three evolutionarily conserved nuclear genes, A. melanocortin receptor 1 (Mc1r); B. Pro-opiomelanocortin (POMC); and C. recombination-activation gene 1 (RAG-1). For POMC, the first "site" represents a covarying doublet of nucleotides. Not every individual could be scored for every site in each gene. Es represents an individual with an "*E. sosorum*" mitochondrial haplotype, En an *E. nana* haplotype, Er an *E. rathbuni* haplotype, and Ew an *E. waterlooensis* haplotype.

A. Mc1r

Species/ population	Site 1 CC	TT	CT	Site 2 GG	TT	GT
E. sosorum	17/21 Es=12 En=3 Ew=2	2/21 Es=2 En=0 Ew=0	2/21 Es=2 En=0 Ew=0	1/16 Es=1 En=0 Ew=0	12/16 Es=8 En=3 Ew=1	3/16 Es=2 En=1 Ew=0
Taylor Springs	8/8	0/8	0/8	0/8	8/8	0/8
Spillar Spring	10/10	0/10	0/10	0/7	7/7	0/7
Cold Spring	3/5	0/5	2/5	0/5	3/5	2/5
Blowing Sink	0/9	1/9	8/9	9/9	0/9	0/9
E. nana	14/14	0/14	0/14	14/14	0/14	0/14
E. waterlooensis	4/14 Ew=2 Er=3 Es=0	4/14 Ew=0 Er=3 Es=1	6/14 Ew=2 Er=4 Es=0	11/13 Ew=4 Er=7 Es=0	0/13	2/13 Ew=1 Er=0 Es=1

B. POMC

Species/ population	"Site" 1 AA/TT	AT/GT	TT/GG	Site 2 AA	GG	AG
E. sosorum	4/54 Es=3 En=0 Ew=1	15/54 Es=12 En=2 Ew=1	35/54 Es=22 En=13 Ew=0	21/56 Es=10 En=11 Ew=0	17/56 Es=15 En=1 Ew=1	18/56 Es=13 En=4 Ew=1
Taylor Springs	0/9	0/9	9/9	9/9	0/9	0/9
Spillar Spring	0/6	0/6	6/6	6/6	0/6	0/6
Cold Spring	0/4	1/4	3/4	2/5	2/5	1/5
Blowing Sink	0/8	3/8	5/8	6/9	0/9	3/9
E. nana	0/23	0/23	23/23	23/23	0/23	0/23
E. waterlooensis	8/12 Ew=1 Er=7 Es=0	4/12 Ew=2 Er=1 Es=1	0/12	0/14	10/12 Ew=3 Er=6 Es=1	2/12 Ew=1 Er=1 Es=0

Table 1 continued.

C. RAG-1

Species/ population	Site 1 CC	TT	CT	Site 2 AA	GG	AG
E. sosorum	1/54 Es=1 En=0 Ew=0	36/54 Es=28 En=15 Ew=1	15/54 Es=11 En=3 Ew=1	36/54 Es=28 En=15 Ew=1	1/54 Es=1 En=0 Ew=0	15/54 Es=11 En=3 Ew=1
Taylor Springs	0/8	8/8	0/8	8/8	0/8	0/8
Spillar Spring	0/8	8/8	0/8	8/8	0/8	0/8
Cold Spring	0/3	3/3	0/3	3/3	0/3	0/3
Blowing Sink	0/7	7/7	0/7	7/7	0/7	0/7
E. nana	0/24	24/24	0/24	24/24	0/24	0/24
E. waterlooensis	13/13 Ew=4 Er=8 Es=1	0/13	0/13	0/13	12/12 Ew=3 Er=8 Es=1	0/13

Species/ population	Site 3 AA	GG	AG	Site 4 CC	TT	CT
E. sosorum	38/54 Es=26 En=11 Ew=1	1/54 Es=1 En=0 Ew=0	15/54 Es=11 En=3 Ew=1	53/53 Es=33 En=18 Ew=2	0/53	0/53
Taylor Springs	8/8	0/8	0/8	8/8	0/8	0/8
Spillar Spring	8/8	0/8	0/8	8/8	0/8	0/8
Cold Spring	3/3	0/3	0/3	3/3	0/3	0/3
Blowing Sink	7/7	0/7	0/7	7/7	0/7	0/7
E. nana	24/24	0/24	0/24	0/24	24/24	0/24
E. waterlooensis	0/12	11/11 Ew=2 Er=8 Es=1	0/12	13/13 Ew=4 Er=8 Es=1	0/13	0/13

To next page for continuation of RAG-1.

C. Table 1. RAG-1 continued

Species/ population	Site 5 AA	TT	AT
E. sosorum	36/48 Es=24 En=11 Ew=1	1/48 Es=1 En=0 Ew=0	11/48 Es=7 En=3 Ew=1
Taylor Springs	7/7	0/7	0/7
Spillar Spring	8/8	0/8	0/8
Cold Spring	3/3	0/3	0/3
Blowing Sink	6/6	0/6	0/6
E. nana	24/24	0/24	0/24
E. waterlooensis	0/12	11/11 Ew=2 Er=8 Es=1	0/12

Table 2. Observed average heterozygosity by microsatellite locus and population. Values are percentages.

Species/ population	Locus A4v2	Locus u6	Locus u9	Locus u27	Locus u55
E. sosorum	82.1	86.1	53.6	33.3	28.8
Taylor Springs	0.0	0.0	20.0	0.0	22.0
Spillar Spring	50.0	50.0	71.4	48.6	50.0
Cold Spring	54.0	60.0	100.0	20.0	75.0
Blowing Sink	41.7	33.3	33.3	23.1	30.8
E. nana	35.7	67.6	16.7	54.1	70.6
E. waterlooensis	45.5	54.5	0.0	12.6	100.0

Table 3. A. Pairwise F_{ST} values (below diagonal) and estimated levels of gene flow (Nm, number of migrants per generation; above diagonal) based on five variable microsatellite loci for all populations and species examined. B. Pairwise comparisons based on R_{ST} values. *E. sosorum* refers to the population at the type locality, Barton Springs, *E. nana* refers to the population at the type locality, San Marcos Springs, and *E. waterlooensis* refers to the population at the type locality, Barton Springs. The other populations are geographically peripheral or intermediate.

A.

Species/ population	*E. sosorum*	Taylor Springs	Spillar Spring	Cold Spring	Blowing Sink	*E. nana*	*E. waterlooensis*
E. sosorum	0	0.588	1.907	3.375	3.575	1.436	1.309
Taylor Springs	0.298	0	0.932	0.453	0.506	0.301	0.263
Spillar Spring	0.196	0.212	0	1.211	1.430	0.741	0.645
Cold Spring	0.069	0.356	0.171	0	1.414	0.851	0.936
Blowing Sink	0.065	0.331	0.149	0.150	0	1.385	0.929
E. nana	0.148	0.454	0.252	0.227	0.153	0	1.235
E. waterlooensis	0.160	0.487	0.279	0.213	0.212	0.168	0

B.

Species/ population	*E. sosorum*	Taylor Springs	Spillar Spring	Cold Spring	Blowing Sink	*E. nana*	*E. waterlooensis*
E. sosorum	0	0.941	1.800	3.231	11.551	0.132	0.119
Taylor Springs	0.210	0	0.586	0.086	0.206	0.182	0.050
Spillar Spring	0.122	0.299	0	0.206	0.712	0.207	0.067
Cold Spring	0.072	0.744	0.548	0	0.614	0.215	0.157
Blowing Sink	0.021	0.548	0.260	0.289	0	0.169	0.199
E. nana	0.655	0.578	0.547	0.538	0.596	0	0.067
E. waterlooensis	0.677	0.832	0.790	0.614	0.677	0.789	0

Table 4. Proportion of total molecular variance, partitioned within versus among species and populations based on AMOVA analyses of microsatellite data using F_{st} and R_{st} values.

	Within populations/species	Among populations/species
F_{st}	73%	27%
R_{st}	36%	64%

Table 5. Raw microsatellite data for populations and species. Note that, particularly for *E.nana*, there are considerable missing data and some of these results need to be confirmed by replication. Bold headings indicate the microsatellite locus, and allelic identities by fragment size are indicated as two-column diploid genotypes. Individuals of *E. sosorum* ("Esos") from Barton Springs that possess *E. nana*-like mt haplotypes are indicated in the rightmost column by En; those with *E. waterlooensis*-like haplotypes are indicated by Ew. The individual of *E. waterlooensis* that possesses an *E. sosorum*-like haplotype is indicated by Es, and those with haplotypes most similar to that of E. rathbuni 1 are designated Er. Missing data are indicated by 0.0. Allele sizes have been rounded to within +/- 1 bp for consistency of identification.

Population	**A4v2**		**u6**		**u9**		**u27**		**u55**		
EsosE1	352.0	355.0	273.0	280.0	128.0	132.0	192.0	192.0	267.0	267.0	
EsosE2	340.0	348.0	254.0	273.0	140.0	140.0	192.0	192.0	267.0	267.0	
EsosE3	350.0	356.0	0.0	273.0	132.0	132.0	192.0	203.0	263.0	267.0	
EsosE4	352.0	352.0	246.0	273.0	128.0	132.0	176.0	179.0	267.0	284.0	
EsosE6	311.0	311.0	249.0	273.0	128.0	128.0	176.0	176.0	259.0	259.0	
EsosE7	340.0	352.0	280.0	280.0	128.0	132.0	192.0	192.0	267.0	267.0	En
EsosE8	344.0	352.0	229.0	264.0	132.0	132.0	196.0	196.0	267.0	267.0	
EsosE9	340.0	360.0	273.0	291.0	132.0	132.0	196.0	196.0	267.0	267.0	
EsosE10	350.0	350.0	261.0	273.0	132.0	132.0	192.0	192.0	267.0	267.0	
EsosE11	340.0	355.0	0.0	0.0	128.0	136.0	192.0	192.0	267.0	267.0	
EsosE12	340.0	352.0	268.0	273.0	128.0	132.0	178.0	192.0	267.0	267.0	
EsosE13	340.0	355.0	254.0	264.0	128.0	136.0	0.0	0.0	259.0	267.0	
EsosE14	336.0	352.0	261.0	273.0	120.0	132.0	188.0	192.0	267.0	267.0	En
EsosE15	336.0	344.0	268.0	280.0	128.0	132.0	192.0	196.0	267.0	267.0	
EsosE16	344.0	344.0	254.0	273.0	132.0	132.0	188.0	192.0	259.0	267.0	
EsosE17	340.0	340.0	246.0	268.0	128.0	132.0	192.0	192.0	267.0	267.0	
EsosE18	352.0	352.0	254.0	273.0	128.0	128.0	196.0	196.0	259.0	267.0	
EsosE19	344.0	350.0	268.0	284.0	132.0	132.0	192.0	192.0	259.0	267.0	
EsosE20	340.0	350.0	261.0	273.0	128.0	136.0	192.0	192.0	267.0	267.0	
EsosE21	340.0	352.0	229.0	254.0	128.0	132.0	192.0	192.0	267.0	267.0	En
EsosE22	340.0	348.0	264.0	273.0	132.0	132.0	200.0	212.0	267.0	267.0	En
EsosE23	0.0	0.0	229.0	229.0	128.0	132.0	192.0	192.0	259.0	267.0	
EsosE24	340.0	355.0	246.0	273.0	128.0	132.0	192.0	200.0	267.0	267.0	En
EsosE25	340.0	348.0	273.0	273.0	132.0	132.0	192.0	192.0	267.0	287.0	
EsosE26	344.0	352.0	273.0	280.0	128.0	132.0	192.0	192.0	259.0	267.0	
EsosE27	340.0	352.0	246.0	280.0	132.0	132.0	188.0	188.0	267.0	267.0	
EsosE28	348.0	352.0	246.0	273.0	128.0	128.0	192.0	192.0	267.0	267.0	
EsosE29	340.0	344.0	268.0	268.0	128.0	128.0	192.0	192.0	259.0	267.0	
EsosE30	340.0	348.0	264.0	268.0	128.0	128.0	180.0	180.0	267.0	267.0	
EsosE31	344.0	355.0	273.0	273.0	128.0	132.0	176.0	176.0	267.0	267.0	
EsosE32	332.0	340.0	260.0	264.0	136.0	136.0	192.0	192.0	259.0	267.0	
EsosE33	332.0	340.0	268.0	280.0	136.0	136.0	192.0	200.0	267.0	267.0	
EsosE34	340.0	348.0	254.0	280.0	128.0	132.0	188.0	192.0	267.0	267.0	

EsosE35	344.0	344.0	246.0	273.0	128.0	132.0	176.0	188.0	0.0	0.0
EsosE36	344.0	344.0	246.0	273.0	128.0	132.0	188.0	188.0	267.0	267.0
EsosE37	340.0	344.0	268.0	280.0	120.0	132.0	0.0	0.0	267.0	267.0
EsosE38	340.0	344.0	273.0	280.0	120.0	132.0	0.0	0.0	0.0	0.0En
EsosE39	340.0	344.0	261.0	273.0	128.0	128.0	188.0	192.0	263.0	263.0En
EsosP1	311.0	352.0	276.0	291.0	136.0	136.0	176.0	176.0	267.0	267.0
EsosP2	340.0	355.0	273.0	280.0	132.0	132.0	192.0	192.0	267.0	267.0
EsosP3	311.0	348.0	246.0	280.0	132.0	136.0	184.0	196.0	267.0	267.0En
EsosP4	348.0	360.0	264.0	273.0	132.0	132.0	192.0	192.0	267.0	267.0
EsosP5	348.0	348.0	280.0	280.0	132.0	136.0	188.0	192.0	267.0	267.0En
EsosP6	340.0	348.0	261.0	280.0	120.0	136.0	188.0	188.0	267.0	267.0En
EsosP7	0.0	0.0	261.0	280.0	128.0	132.0	188.0	188.0	267.0	267.0En
EsosP8	340.0	352.0	273.0	280.0	132.0	136.0	176.0	196.0	267.0	267.0
EsosP9	340.0	352.0	246.0	254.0	128.0	132.0	172.0	176.0	267.0	267.0En
EsosP10	340.0	344.0	0.0	0.0	128.0	132.0	188.0	188.0	267.0	267.0
EsosP11	340.0	352.0	246.0	264.0	128.0	136.0	176.0	176.0	267.0	267.0Ew
EsosP12	336.0	340.0	273.0	276.0	132.0	132.0	192.0	192.0	259.0	267.0
EsosP13	340.0	352.0	229.0	295.0	128.0	132.0	176.0	176.0	267.0	267.0
EsosP14	311.0	340.0	268.0	268.0	128.0	132.0	192.0	192.0	267.0	267.0
EsosP15	352.0	356.0	264.0	276.0	132.0	132.0	188.0	192.0	259.0	267.0
EsosP16	340.0	340.0	264.0	273.0	132.0	132.0	192.0	192.0	267.0	267.0
EsosP17	311.0	340.0	229.0	264.0	128.0	132.0	176.0	176.0	259.0	267.0Ew
EsosP18	344.0	348.0	229.0	280.0	132.0	136.0	192.0	192.0	0.0	0.0En
EsosP19	311.0	340.0	264.0	273.0	128.0	128.0	192.0	192.0	267.0	267.0
EsosP20	340.0	340.0	246.0	273.0	128.0	128.0	192.0	192.0	240.0	267.0
EsosU1	340.0	340.0	264.0	273.0	136.0	136.0	196.0	196.0	259.0	267.0
EsosU2	332.0	334.0	254.0	280.0	132.0	136.0	196.0	200.0	259.0	267.0En
EsosU3	311.0	340.0	254.0	254.0	132.0	136.0	192.0	196.0	259.0	259.0En
EsosU4	332.0	352.0	254.0	254.0	132.0	132.0	192.0	200.0	267.0	267.0
EsosU5	311.0	352.0	254.0	364.0	132.0	136.0	192.0	196.0	259.0	267.0En
EsosZ1	340.0	360.0	229.0	254.0	132.0	132.0	196.0	196.0	267.0	267.0
EsosZ2	340.0	352.0	276.0	280.0	132.0	132.0	192.0	200.0	267.0	267.0
EsosZ3	340.0	344.0	246.0	273.0	136.0	136.0	176.0	176.0	267.0	267.0En
EsosZ4	340.0	344.0	268.0	280.0	132.0	132.0	188.0	188.0	259.0	267.0
EsosZ5	340.0	352.0	273.0	273.0	136.0	140.0	192.0	192.0	267.0	267.0
EsosZ6	311.0	348.0	254.0	280.0	132.0	136.0	192.0	192.0	267.0	267.0
Taylor1902	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	267.0	267.0
Taylor1903	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	267.0	267.0
Taylor1904	0.0	0.0	280.0	280.0	136.0	136.0	188.0	188.0	256.0	267.0
Taylor1905	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	267.0	267.0
Taylor1906	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	256.0	267.0
Taylor1907	348.0	348.0	280.0	280.0	136.0	140.0	188.0	188.0	267.0	267.0
Taylor1908	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	267.0	267.0
Taylor1909	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	0.0	0.0

Taylor1910	348.0	348.0	280.0	280.0	136.0	136.0	188.0	188.0	267.0	267.0
Taylor1911	0.0	0.0	280.0	280.0	136.0	140.0	188.0	188.0	267.0	267.0
Spillar2028	0.0	0.0	276.0	280.0	136.0	136.0	188.0	192.0	267.0	267.0
Spillar2029	348.0	352.0	0.0	0.0	124.0	136.0	0.0	192.0	256.0	267.0
Spillar2030	348.0	352.0	280.0	280.0	124.0	136.0	188.0	192.0	256.0	267.0
Spillar2031	348.0	348.0	0.0	0.0	124.0	124.0	188.0	192.0	256.0	267.0
Spillar2032	348.0	348.0	268.0	268.0	124.0	136.0	188.0	192.0	267.0	267.0
Spillar2033	0.0	0.0	0.0	280.0	124.0	136.0	192.0	192.0	267.0	267.0
Spillar2034	348.0	348.0	276.0	288.0	124.0	136.0	188.0	192.0	267.0	267.0
Spillar2035	340.0	348.0	276.0	0.0	0.0	0.0	0.0	0.0	256.0	267.0
ColdSpr76	311.0	340.0	254.0	280.0	132.0	136.0	192.0	192.0	267.0	267.0
ColdSpr77	340.0	340.0	251.0	273.0	132.0	136.0	208.0	208.0	259.0	267.0
ColdSpr78	340.0	348.0	251.0	251.0	132.0	136.0	196.0	196.0	259.0	267.0
ColdSpr1924	340.0	348.0	254.0	254.0	128.0	0.0	196.0	196.0	259.0	267.0
ColdSpr1925	340.0	348.0	254.0	268.0	0.0	136.0	192.0	196.0	0.0	267.0
BlSink1125	336.0	336.0	0.0	0.0	136.0	136.0	192.0	196.0	259.0	267.0
BlSink1769	336.0	344.0	273.0	273.0	128.0	128.0	192.0	196.0	259.0	267.0
BlSink1770	344.0	344.0	280.0	280.0	128.0	136.0	192.0	192.0	267.0	267.0
BlSink1771	336.0	336.0	0.0	280.0	128.0	136.0	0.0	0.0	267.0	267.0
BlSink1772	336.0	348.0	261.0	273.0	136.0	136.0	188.0	188.0	267.0	267.0
BlSink1773	336.0	344.0	0.0	0.0	136.0	136.0	192.0	192.0	259.0	267.0
BlSink1832	336.0	336.0	254.0	273.0	128.0	136.0	188.0	188.0	267.0	267.0
BlSink1833	336.0	336.0	261.0	261.0	128.0	128.0	192.0	192.0	267.0	267.0
BlSink1834	0.0	0.0	268.0	268.0	128.0	136.0	188.0	192.0	0.0	0.0
BlSink1835	344.0	352.0	261.0	280.0	0.0	0.0	196.0	196.0	267.0	267.0
BlSink1836	336.0	336.0	261.0	261.0	128.0	128.0	192.0	192.0	267.0	267.0
BlSink1912	336.0	348.0	254.0	261.0	132.0	136.0	192.0	192.0	267.0	267.0
BlSink1913	336.0	0.0	280.0	280.0	128.0	128.0	192.0	192.0	0.0	267.0
BlSink1926	0.0	0.0	284.0	284.0	128.0	128.0	0.0	0.0	267.0	267.0
BlSink1927	0.0	0.0	280.0	280.0	128.0	128.0	196.0	196.0	0.0	267.0
BlSink1928	344.0	344.0	0.0	0.0	128.0	128.0	0.0	0.0	259.0	267.0
Enana116	352.0	355.0	0.0	0.0	128.0	128.0	0.0	0.0	276.0	276.0
Enana117	355.0	355.0	254.0	268.0	128.0	0.0	230.0	230.0	267.0	267.0
Enana119	0.0	0.0	261.0	0.0	128.0	128.0	242.0	0.0	267.0	276.0
Enana120	355.0	355.0	261.0	261.0	128.0	128.0	188.0	192.0	280.0	309.0
Enana121	355.0	375.0	261.0	273.0	128.0	128.0	242.0	242.0	273.0	309.0
Enana122	398.0	414.0	265.0	273.0	128.0	128.0	225.0	225.0	280.0	283.0
Enana123	375.0	390.0	265.0	273.0	128.0	128.0	230.0	0.0	0.0	0.0
Enana124	0.0	0.0	273.0	273.0	128.0	136.0	192.0	230.0	267.0	333.0
Enana125	0.0	0.0	0.0	0.0	0.0	0.0	242.0	242.0	0.0	0.0
Enana126	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Enana127	390.0	390.0	0.0	0.0	128.0	136.0	230.0	234.0	259.0	272.0
Enana128	355.0	355.0	276.0	284.0	128.0	128.0	230.0	240.0	280.0	333.0
Enana129	0.0	0.0	276.0	284.0	128.0	128.0	242.0	242.0	280.0	280.0

Enana130	375.0	414.0	273.0	273.0	128.0	128.0	230.0	238.0	272.0	272.0
Enana132	355.0	355.0	268.0	268.0	128.0	128.0	230.0	230.0	0.0	0.0
Enana133	414.0	414.0	273.0	311.0	128.0	136.0	234.0	257.0	267.0	333.0
Enana134	355.0	355.0	268.0	315.0	128.0	128.0	230.0	230.0	276.0	280.0
Enana135	355.0	355.0	268.0	311.0	128.0	128.0	230.0	234.0	305.0	305.0
Enana136	375.0	390.0	265.0	273.0	128.0	128.0	230.0	238.0	269.0	269.0
Enana137	355.0	355.0	273.0	273.0	128.0	128.0	230.0	230.0	0.0	0.0
Enana138	398.0	0.0	273.0	273.0	128.0	128.0	230.0	242.0	267.0	280.0
Enana139	367.0	375.0	311.0	311.0	128.0	132.0	230.0	230.0	267.0	333.0
Enana140	375.0	375.0	261.0	261.0	0.0	0.0	0.0	0.0	333.0	333.0
Enana141	0.0	0.0	268.0	273.0	128.0	128.0	176.0	192.0	276.0	280.0
Enana142	375.0	375.0	259.0	259.0	128.0	136.0	0.0	0.0	267.0	267.0
Enana143	390.0	0.0	259.0	273.0	128.0	128.0	230.0	234.0	325.0	329.0
Enana248	355.0	355.0	265.0	265.0	0.0	0.0	234.0	242.0	0.0	0.0
Enana249	355.0	355.0	273.0	276.0	128.0	128.0	200.0	230.0	302.0	333.0
Enana250	372.0	372.0	273.0	273.0	128.0	128.0	230.0	230.0	267.0	0.0
Enana251	0.0	0.0	0.0	0.0	0.0	0.0	230.0	230.0	309.0	325.0
Enana252	355.0	355.0	261.0	273.0	128.0	128.0	230.0	230.0	286.0	286.0
Enana253	355.0	0.0	254.0	273.0	128.0	128.0	204.0	230.0	267.0	302.0
Enana254	388.0	388.0	261.0	273.0	128.0	132.0	192.0	230.0	276.0	313.0
Enana255	360.0	360.0	273.0	273.0	128.0	128.0	208.0	236.0	313.0	0.0
Enana256	360.0	0.0	261.0	273.0	128.0	128.0	230.0	230.0	280.0	305.0
Enana257	0.0	0.0	268.0	273.0	128.0	128.0	212.0	230.0	325.0	337.0
Enana258	360.0	384.0	261.0	273.0	128.0	128.0	230.0	230.0	0.0	0.0
Enana259	380.0	384.0	259.0	273.0	0.0	0.0	236.0	240.0	302.0	309.0
Enana260	352.0	375.0	261.0	268.0	128.0	128.0	232.0	240.0	280.0	302.0
Enana261	0.0	0.0	268.0	273.0	128.0	128.0	236.0	236.0	276.0	302.0
Enana262	360.0	0.0	265.0	273.0	128.0	128.0	238.0	238.0	309.0	325.0
Enana263	0.0	0.0	254.0	273.0	128.0	128.0	192.0	230.0	325.0	329.0
Enana264	360.0	360.0	261.0	273.0	128.0	128.0	230.0	230.0	272.0	272.0
Ewat1993	311.0	394.0	229.0	0.0	136.0	136.0	172.0	172.0	236.0	267.0
Ewat1994	311.0	311.0	229.0	242.0	128.0	128.0	172.0	176.0	240.0	259.0Er
Ewat1995	307.0	344.0	229.0	246.0	132.0	132.0	176.0	176.0	240.0	259.0Er
Ewat1996	311.0	315.0	229.0	242.0	136.0	136.0	176.0	176.0	259.0	263.0Er
Ewat1997	311.0	315.0	229.0	246.0	0.0	0.0	172.0	176.0	240.0	259.0Er
Ewat1998	0.0	0.0	242.0	246.0	128.0	128.0	0.0	0.0	232.0	240.0
Ewat1999	311.0	311.0	229.0	229.0	128.0	128.0	0.0	0.0	240.0	259.0Er
Ewat2000	311.0	311.0	229.0	246.0	128.0	128.0	172.0	176.0	236.0	259.0Er
Ewat2001	311.0	311.0	229.0	229.0	128.0	0.0	0.0	0.0	240.0	259.0
Ewat2002	311.0	311.0	229.0	229.0	128.0	0.0	172.0	176.0	240.0	259.0Es
Ewat2003	311.0	311.0	229.0	229.0	132.0	132.0	192.0	192.0	232.0	240.0Er
Ewat2004	315.0	340.0	229.0	229.0	128.0	128.0	172.0	196.0	240.0	259.0