Genetic characterization of *Eurycea* salamanders from Jacob's Well, Hays County, Texas

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by

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Abstract

Introduction

The genus *Eurycea* Rafinesque, 1822 contains salamander species from across the USA, with most species occurring in cave or spring habitats. The genus is known for taxonomic uncertainty, usually associated with limited specimen availability often directly consequent of small geographic distributions and difficulty in accessing those habitats by researchers. The group also shows a high morphological variability with some forms once considered unique enough to warrant distinction as monotypic genera (i.e. *Typhlomolge* and *Haideotriton*). Recent investigations of *Eurycea* from central Texas have concluded that many species are represented where previously only a few species had been previously distinguished (Chippendale et al. 2000; Hillis et al. 2001).

The results to date from these studies and others have not resolved many questions surrounding the genetic divergence among populations, the potential for gene flow among those populations, or the applicability of assigning species rank to each spring head population. Alternative hypotheses have been tested to discern above- or below-ground connectivity (Lucas et al. 2009) with those authors concluding that the populations are isolated and should be treated as such.

The geology of central Texas is dominated by its karst topography. As the limestone has dissolved, the subterranean habitats, and springs that provide the habitats for these salamanders, have become available. The region is characterized by historically abundant surface springs, large cave systems, and dramatic rainfall events. With increased urbanization, surface water impoundment, and ground-water withdrawals, the original ecosystem structure for these salamanders has been altered significantly in the last century. Today, only the largest springs maintain outflows and many of those sites are potentially at risk from ongoing development or other anthropogenic impacts.

One well known site is Jacob's Well outside of Wimberley, Texas. This site is noted as the longest underwater cave in Texas and for its consistent outflow from the aquifer. It represents one of the primary inflows supporting the Blanco River and continued to flow throughout the drought of record during the 1950s. The spring has stopped flowing twice in recent times; the first was in 2000 and second in 2008. The site has recently been documented to contain *Eurycea*.

The purpose of this study was to characterize the mtDNA variation for salamanders collected from Jacob's Well by Zara Environmental, Inc.

Materials and Methods

Individuals were sampled across central Texas (Appendix A), nonconsumptively where possible. Forty whole specimen, 75 tail tip, 3 liver, 3 skin, 1 muscle, 1 heart, and 4 unknown tissue samples were collected and stored in 70% ethanol at -80°C. Tissues were deposited in the Michael R. J. Forstner Frozen Tissue catalog at Texas State University–San Marcos.

Eurycea were sampled under Department of the Interior, U.S. Fish and Wildlife Service, Federal Fish and Wildlife Permit Number TE676811-0 and Texas Parks and Wildlife Scientific Permit Numbers SPR-0102-191, SPR-0290-022, and SPR-0390-045 and under Institutional Animal Care and Use Committee approvals 0715_0428_07, 04-3D2AAE71, 04-046 E25 EBSA, and 1010_0501_09.

DNA was isolated from tissue (1-2 mm^3) using a DNeasy® Tissue Kit (QIAGEN Inc.). A partial sequence of the mitochondrial cytochrome b gene was sequenced. Amplification was performed using the primers MVZ15 (Chippindale et al. 2000) and EURCB9 (Hillis et al. 2001) in 50 µl reactions with 4 mM $MgCl_2$, 0.1 mM dNTPs, 0.01 µM each primer, 2.5 units GoTaq® Flexi DNA polymerase (Promega), and pH = 8.5. PCR was performed with an initial denaturing period of 95°C for 5 min then 35 cycles, each consisting of denaturing at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension period of 72°C for 5 min. PCR products were purified with an AMPure® PCR Purification System (Agencourt Bioscience Corporation), and then cycle sequenced with the above primers, using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Thermal cycling was performed with an initial denaturing period of 96°C for 1 min then 25 cycles, each consisting of 96°C for 1 min, 50°C for 5 sec, and 60°C for 4 min. Products were cleaned by ethanol precipitation and analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems). Resultant sequences were edited and aligned in SEQUENCHER™ 4.5 (Gene Codes Corp.).

To assess the phylogenetic relationships within central Texas *Eurycea*, maximum likelihood (ML, Felsenstein 1981), neighbor joining (NJ), and Bayesian analyses using mtDNA data were performed. In addition to sequences generated by us, 22 GenBank accessions were included in analyses (Table 1). *Eurycea multiplicata* (GenBank AY014854) was used as an outgroup (Chippindale et al. 2000). Model parameters for maximum likelihood, which were estimated by hLRT and AIC using MODELTEST 3.7 (Posada & Crandall 1998), were used as input in a ML heuristic search in PAUP* 4.0b10 (Swofford 2002). Neighbor joining topologies were generated using HKY85 in PAUP* 4.0b10. Bootstrap values (Felsenstein 1985) were estimated from 1,000 replicates in a neighbor-joining search in PAUP* 4.0b10 for NJ analysis. Parameters of a best-fit nucleotide model of evolution for Bayesian analysis were determined by hLRT and AIC in MRMODELTEST 2.0 (Nylander 2004), and MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) was implemented for one million generations, saving every hundredth tree, and with a burn-in of 100,000 trees. Similar analyses were also conducted on a reduced dataset (see Results).

To assess populational relationships, a statistical parsimony network (Templeton et al. 1992) of mtDNA haplotypes was constructed using TCS 1.21 (Clement et al. 2000), with a connection limit of 30 and gaps treated as a 5th state. Differences in allele frequencies among sites were assessed by computing pairwise F_{ST} values with 10,000 permutations and a significance value of 0.05 and by performing Fisher's exact test of population differentiation in ARLEQUIN 3.11 (Excoffier et al. 2005). Isolation-by-distance was tested among individuals with a Mantel test (Mantel 1967) in ALLELES IN SPACE 1.0 (AIS, Miller 2005) with 1,000 permutations.

Results

One hundred twenty-seven *Eurycea* in seven counties were sampled for this study (Table 1 and Appendix A). The 1026-bp cytochrome b alignment of 127 individuals resulted in 26 unique haplotypes (GenBank Accession Nos. HQ713576-HQ713601); with the addition of already published GenBank accessions, the number of individuals was 149 and of unique haplotypes was 44. The model of evolution that best fitted the data was TVM+G (chosen by AIC) and HKY+G (chosen by hLRT) as determined by MODELTEST and GTR+G chosen by AIC and hLRT as determined by MRMODELTEST. The Bayesian phylogram is shown in Fig. 1. NJ, ML, and Bayesian analyses resulted in similar topologies. Similar analyses were performed on a reduced dataset, i.e., only individuals recovered in clade 2 from Fig. 1. HKY+G (chosen by AIC and hLRT) was the model of evolution that best fitted the data as determined by MRMODELTEST; the resultant Bayesian phylogram is shown in Fig. 2.

The statistical parsimony network of 24 unique mtDNA haplotypes in 116 individuals is presented in Fig. 3. Three haplotypes were detected at Jacob's Well; all were unique to that site. In fact, 12 haplotypes were found at only one site: A, B, C, D, E, F, H, I, J, K, L, M, N, and O (Table 2). Three haplotypes were found at multiple sites: G, L, and M (Table 2).

Pairwise F_{ST} values were calculated for groups of individuals recovered in clade 2 from Fig. 1 (Table 3). Most values were not significant, which is likely due to the inclusion of already published GenBank accessions that had very low sample sizes. Very high F_{ST} values were found for *E. nana* (F_{ST} = 0.927-0.974) and between a clade containing *E. pterophila* and one containing *E. neotenes* and *E.* 'Comal Springs' sp. (F_{ST} = 0.849-1.000). Within the clade containing *E. neotenes* and *E.* 'Comal Springs' sp., F_{ST} values were 0.356-0.768. Within the clade containing *E. pterophila*, F_{ST} values were 0.571-0.792. Significant genetic differentiation was detected using Fisher's exact test for 22 combinations of sites/species (Table 3). Jacob's Well was different from the other locations within the clade that contains *E. pterophila* sites (Fern Bank Springs and Ott's Spring). All three were different from Comal Springs and Hueco Springs. Again, most tests were not significant, but could be explained by low sample sizes. The Mantel test of only '*neotenes*' and '*pterophila*' individuals (those with haplotypes A, B, C, D, E, F, G,

H, I, J , and K; $n = 100$) revealed significant positive, and large, correlations between genetic distances and geographic distance (i.e., isolation-by-distance) ($r = 0.888$, $P < 0.001$).

Discussion

The unique environmental context of Texas has created tremendous biodiversity both above and below its surface. Texas ranks second behind only California in total diversity, third in total endemism, but also fourth in total extinctions across the USA (Stein 2002). Texas is also fifth in total amphibian diversity, with at least 20% of those species at risk placing it among the top ten for total percentage of amphibian taxa at risk (Stein 2002). The endemic salamander fauna of central Texas has some of the smallest depicted distributions for any amphibian in the United States. These taxa are poorly known, often poorly documented, and provide a confusing array of phenotypes and morphology even among a single spring site. Seemingly the resolution to this would be genetic data, but the variability extends to the genetic results.

One reason for the current levels of uncertainty is simply the novelty of the investigations for these taxa. With the majority of "new" *Eurycea* species having been described in the last few decades, reviews and revisions to the alpha taxonomy of the group have not yet been conducted. The achievement of a stable evolutionary taxonomy supported by evolutionary relationships is the goal, but it cannot be achieved quickly given the diversity, number of sites, and the often contradictory conclusions of systematists examining the data.

We sought a specific answer in our evaluation of the salamanders from Jacob's Well. Does this location contain a unique lineage of evolutionarily distinct *Eurycea*? The answer to that question pragmatically must include context among other populations, previous studies, and additional data from specimens outside of the study site. In our work we first chose to use an mtDNA marker in order to provide higher resolution than would be possible with similar amounts of nuclear DNA sequence data. We chose to use sequences from cytochrome b (Chippendale et al. 2000), because the database for homologous sequences is larger than that for ND4 (Lucas et al. 2009).

The results demonstrate the underlying instability of the current taxonomy, at least from the perspective of mtDNA marker analyses (Fig. 1). The resulting topology is in close general agreement with previously published phylogenetic relationships (Chippendale et al. 2000; Lucas et al. 2009). The Texas *Eurycea* resolve two major divisions. The northern group contains *E. chisholmensis*, *E. naufragia*, and *E. tonkawae* which form the sister clade to those occuring south and west (*E. troglodytes, E. nana, E. sosorum, E. latitans, E. tridentifera, E. pterophila*) including the aquifer forms (*E. rathbuni* and *E. waterlooensis*). There are deep genetic divergences between these two sister groups.

Within the northern species group there is a similar deep divergence between *E. naufragia* and the clade containing *E. chisholmensis* and *E. tonkawae*. The southern species

group has much less divergent lineages overall, but discrete units are supported at those lower divergences (Fig. 1). The aquifer-dwelling forms, *E. rathbuni* and *E. waterlooensis*, form a distinct clade that is a sister group to a clade of two groups, *E. pterophila* and *E. troglodytes*. All of the Jacob's Well salamanders fall within the *E. pterophila* clade (Fig. 2).

The samples from Jacob's Well were collected at a variety of depths, including very deep within the cave system (Appendix A). There were no substantial differences among those samples, regardless of depth (Fig. 2). Similarly, there are no substantial differences seen between the samples from Jacob's Well and those from Fern Bank or Ott's Spring (see Lucas et al. 2009 for a map depiction). Furthermore, the data do not resolve any distinction among the Jacob's Well samples and the sequences available for *E. latitans, E. tridentifera,* or *E. pterophila* (Fig. 2). In the evaluation by Lucas et al. (2009), the authors note the presence of unique haplotypes for several sites. While we found some haplotypes that were unique to Jacob's Well and to Ott's Spring, Lucas et al. (2009) found shared haplotypes at these sites for another mtDNA gene, ND4 (Table 2). And, while both studies recovered unique haplotypes at Comal Springs and Hueco Springs, we also found a shared haplotype at those two sites (Table 2).

The actual relationships among the haplotypes for salamanders at these localities are more complex than simply having shared haplotypes or not (Fig. 3). As an example, there are many changes between the haplotype from *E. sosorum*, or that from the sample from the Perdenales river site (Fig. 3) and other clades, but even these seemingly obvious divergences may be more anomalous than representative as these are from a single sample from those locations. The results indicate that the Jacob's Well salamanders are part of a broad group of *E. pterophila* populations and that significantly more work will be required before the current taxonomy can be shown to accurately portray the underlying evolutionary relationships among the salamanders from these localities. Our results do not support the current taxonomic structure for species named within the genus Eurycea occurring in central Texas, instead our results would support a much reduced species taxonomy reflecting the evolutionary relationships depicted by Fig. 1.

It is not clear from any single study how best to interpret the taxonomic decisions that derive from an understanding of evolutionary relationships. The underlying reason that systematics is cyclical is fundamentally a part of the increasing information available for taxonomic groups over time. For example, we compared average genetic distances (uncorrected p) among multiple taxonomic/phylogenetic levels in the genus *Eurycea* (Fig. 4). We sought to examine the overall genetic divergences among described species within the genus. It was our general expectation that comparisons within species would show less genetic variation than between species and that related species groups would follow a similar trend when compared among such groups. This was not the result. The partitioning of genetic variability into names did not follow a recognizable trend for *Eurycea* in Texas. It may simply be that this is not a good way to characterize the populations, but it may also support our contention that Texas *Eurycea*

need significant, comprehensive examination in order to accurately describe the evolutionary variation for this genus.

Further support for the need of comprehensive revision to the taxonomy can be seen in the evaluation of the accessibility to the populations. We chose to illustrate this approach this by simply comparing the straightline geographic distance from Austin, Texas to the type localities for the species examined here. The *E. troglodytes* complex is the furthest from the Capitol building in Austin and encompasses the largest genetic variation (Fig. 4). The answer to the systematics in *Eurycea* is additional work in the entirety of the group, not just in those taxa proximal to the state capitol. Currently, development pressures are not as high further to the west in the area of *E. troglodytes*, but unfortunately that translates to fewer funding dollars available to examine those populations. Ironically, in completing the work for these salamanders as a whole, the results from the areas under the highest development pressures may be more clearly and effectively understood. It is in the context of generic diversity that species composition and evolutionary relationships can be most clearly ascertained.

Jacob's Well is one of a handful of moderately large to large springs that still flow in central Texas (Brune 1975). The reduction of spring flow is a state-wide phenomenon and one that is unlikely to reverse trend. The community-based efforts at Jacob's Well represent the type of conscientious and involved stewardship required to maintain these unique environments in the face of development pressures and human water needs. The salamanders of Jacob's Well do not represent a distinct evolutionary lineage from others in the area based on our analyses of mtDNA sequence data. However, this spring is unique in its large size compared to adjacent localities, it is already stewarded by an engaged community derived effort, and it is an icon for the nearby community of Wimberley, Texas. The conjunction of all of those benefits increase the overall value of this site as a stable locality of *Eurycea* with all the attendant benefits to future research and conservation goals.

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Table 1. Sampling sites by county, number of samples, haplotypes (number of individuals per haplotype), and GenBank Accession Nos. for *Eurycea* individuals used in this study.

Site	*n*	Haplotypes	GenBank	Taxon[a]
Bandera Co.				
Lost Maples Natural Area	6	T (5)	HQ713595	*E.* sp.
		U (1)	HQ713596[b]	
Sutherland Hollow Spring	1	—	AY014853[c]	*E. troglodytes*
Bell Co.				
Salado Springs	1	—	AY014841[c]	*E. chisholmensis*
Bexar Co.				
Helotes Creek Spring	2	—	AY014850[c], AY528400[d]	*E. neotenes*
Comal Co.				
Unknown	1	—	AY260758[e]	*E. latitans*
Comal Springs	1	—	AY260759[e]	*E.* 'Comal Springs' sp.
Comal Springs Run 1	3	G (1)	HQ713582	*E.* sp.
		H (1)	HQ713583,	
		I (1)	HQ713584[b]	
Comal Springs Run 3	3	G (1)		*E.* sp.
		H (1)		
		I (1)		
Honey Creek Cave	1	—	AY014848[c]	*E. tridentifera*
Hueco Springs	6	G (4)		*E.* sp.
		J (1)	HQ713585	
		K (1)	HQ713586[b]	
Ott's Spring	6	D (6)	HQ713579[b]	*E.* sp.
Edwards Co.				
250 m W of RR335, about 8.5 rd km S of jct RR335 & TX Hwy 41	3	V (2)	HQ713597,	*E. troglodytes* complex
		W (1)	HQ713598[b]	
Gillespie Co.				
1.36 rd mi S jct White Oak Rd & Zenner-Alherns Rd on Zenner-Alherns	2	X (1)	HQ713599,	*E.* sp.
		Z (1)	HQ713601[b]	
Trough Spring	1	—	AY014852[c]	*E. troglodytes*
Hays Co.				
Fern Bank Springs	1	—	AY014851[c]	*E. pterophila*
Fern Bank Springs	12	E (11)	HQ713580	*E.* sp.
		F (1)	HQ713581[b]	
Fern Bank Springs, spring-fed stream near Fern Bank Springs	3	E (3)		*E.* sp.
Jacob's Well	24	A (22)	HQ713576	*E.* sp.
		B (2)	HQ713577[b]	
Jacob's Well 20'	10	A (6)		*E.* sp.
		B (4)		
Jacob's Well 20'-70'	12	A (7)		*E.* sp.
		B (5)		
Jacob's Well 70'	7	A (7)		*E.* sp.
Jacob's Well subsurface	14	A (7)		*E.* sp.
		B (5)		
		C (2)	HQ713578[b]	
Rattlesnake Cave	2	—	AY014844, AY014845[c]	*E. rathbuni*
San Marcos Springs	1	—	AY014846[c]	*E. nana*

Spring Lake Below Dam	2	L (1) M (1)	HQ713587 HQ713588[b]	*E. nana*
Spring Lake Diversion Springs	2	M (1) N (1)	 HQ713589[b]	*E. nana*
Spring Lake Hotel Site	2	L (1) O (1)	 HQ713590[b]	*E. nana*
Kendall Co.				
Pfeiffer's Water Cave	1	—	AY014849[c]	*E. latitans*
Kerr Co.				
Stockman Spring	5	Y (5)	HQ713600[b]	*E.* sp.
Polk Co. (Arkansas)				
Band's Spring	1	—	AY014854[c]	*E. multiplicata*
Smith Co.				
Tyler	1	—	AY528401[d]	*E. quadridigitata*
Travis Co.				
Balcones Canyonlands, mainstem above Tributary 7	1	Q (1)	HQ713592[b]	*E. tonkawae*
Balcones Canyonlands, Tributary 5	2	Q (1) R (1)	 HQ713593[b]	*E. tonkawae*
Barton Springs	1	—	AY014857[c]	*E. sosorum*
Barton Springs Pool	1	—	AY014856[c]	*E. waterlooensis*
Hammett's Crossing Spring	1	—	AY014847[c]	*E.* 'Pedernales' sp.
SAS canyon	1	S (1)	HQ713594[b]	*E. tonkawae*
Stillhouse Springs	2	—	AY014842[c], AY691749[f]	*E. tonkawae*
Stillhouse Springs	1	P (1)	HQ713591[b]	*E. tonkawae*
Sunken Garden Spring	1	—	AY014855[c]	*E. waterlooensis*
Williamson Co.				
Cedar Break Hiking Trail Spring	1	—	AY014843[c]	*E. naufragia*

[a]Species as identified in the field or in GenBank.
[b]Data from this study.
[c]Hillis et al. 2001.
[d]Bonett & Chippindale 2004.
[e]Wiens et al. 2003.
[f]Chippindale et al. 2004.

Table 2. Comparison of mtDNA haplotypes (ND4 gene vs. cytochrome b gene) found at nine central Texas sites.

Site	ND4[a]		cytochrome b[b]	
	Haplotype	Number	Haplotype	Number
Haplotypes unique to one site				
Comal Springs	J, K	34, 9	H, I	2, 2
Devil's Backbone	L, M	2, 7	—	—
Fern Bank Springs	E, G, H	32, 4, 1	E, F	14, 1
Hueco Springs	N	13	J, K	1, 1
Jacob's Well	B	2	A, B, C	49, 16, 2
Ott's Spring	D, F	11, 6	D	6
Spring Lake Diversion Springs	—	—	N	1
Spring Lake Hotel Site	—	—	O	1
Haplotypes shared among sites				
Devil's Backbone, Jacob's Well, Ott's Spring	A	9, 20, 1	—	—
Spring Lake Below Dam, Spring Lake Diversion Springs	—	—	M	2
Spring Lake Below Dam, Spring Lake Hotel Site	—	—	L	2
Spring Lake Below Dam, Spring Lake Diversion Springs, Spring Lake Hotel Site	C	29, 32, 31	—	—
Spring Lake Below Dam, Spring Lake Diversion Springs, Spring Lake Hotel Site	I	4, 5, 2	—	—
Comal Springs, Hueco Springs	—	—	G	6

[a]Lucas et al. 2009.
[b]Data from this study.

Table 3. Pairwise F_{ST} values (below diagonal) and *P* values from Fisher's exact test of population differentiation (above diagonal) for 'species' in clade 2 from Fig. 1. Significant F_{ST} values are shown in bold.

	E. 'Pedernales' sp.	*E. nana*	*E. sosorum*	*E. latitans*		*E. tridentifera*
	AY014847	Spring Lake (*n* = 7)	AY014857	AY014849	AY260758	AY104848
AY014847	—	0.496	1.000	1.000	1.000	1.000
Spring Lake	0.954	—	0.493	0.497	0.496	0.503
AY014857	1.000	-0.091	—	1.000	1.000	1.000
AY014849	1.000	0.920	1.000	—	1.000	1.000
AY260758	1.000	0.924	1.000	1.000	—	1.000
AY104848	1.000	0.916	1.000	1.000	1.000	—
Comal Springs	0.959	**0.933**	-0.333	0.817	0.839	0.788
Hueco Springs	0.956	**0.927**	-0.400	0.795	0.821	0.760
AY014850, AY528400	1.000	**0.943**	1.000	1.000	1.000	1.000
Fern Bank Springs	0.972	**0.948**	0.611	0.750	0.869	0.641
Jacob's Well	0.986	**0.974**	0.786	0.869	0.932	0.811
Ott's Spring	1.000	**0.952**	1.000	1.000	1.000	1.000

	E. neotenes? and *E.* 'Comal Springs' sp.			*E. pterophila*?		
	Comal Springs (*n* = 7)	Hueco Springs (*n* = 6)	AY014850, AY528400	Fern Bank Springs (*n* = 16)	Jacob's Well (*n* = 49)	Ott's Spring (*n* = 6)
AY014847	0.245	0.433	0.336	0.171	<0.050	0.143
Spring Lake	<0.050	<0.050	0.165	<0.050	<0.050	<0.050
AY014857	0.244	0.439	0.334	0.162	<0.050	0.142
AY014849	0.252	0.434	0.335	0.170	<0.050	0.141
AY260758	0.256	0.430	0.331	0.175	<0.050	0.143
AY104848	0.248	0.426	0.331	0.156	<0.050	0.142
Comal Springs	—	0.191	0.106	<0.050	<0.050	<0.050
Hueco Springs	**0.356**	—	0.072	<0.050	<0.050	<0.050
AY014850, AY528400	**0.792**	**0.768**	—	<0.050	<0.050	<0.050
Fern Bank Springs	**0.849**	**0.837**	**0.916**	—	<0.050	<0.050
Jacob's Well	**0.913**	**0.906**	**0.953**	**0.792**	—	<0.050
Ott's Spring	**0.864**	**0.855**	**1.000**	**0.571**	**0.719**	—

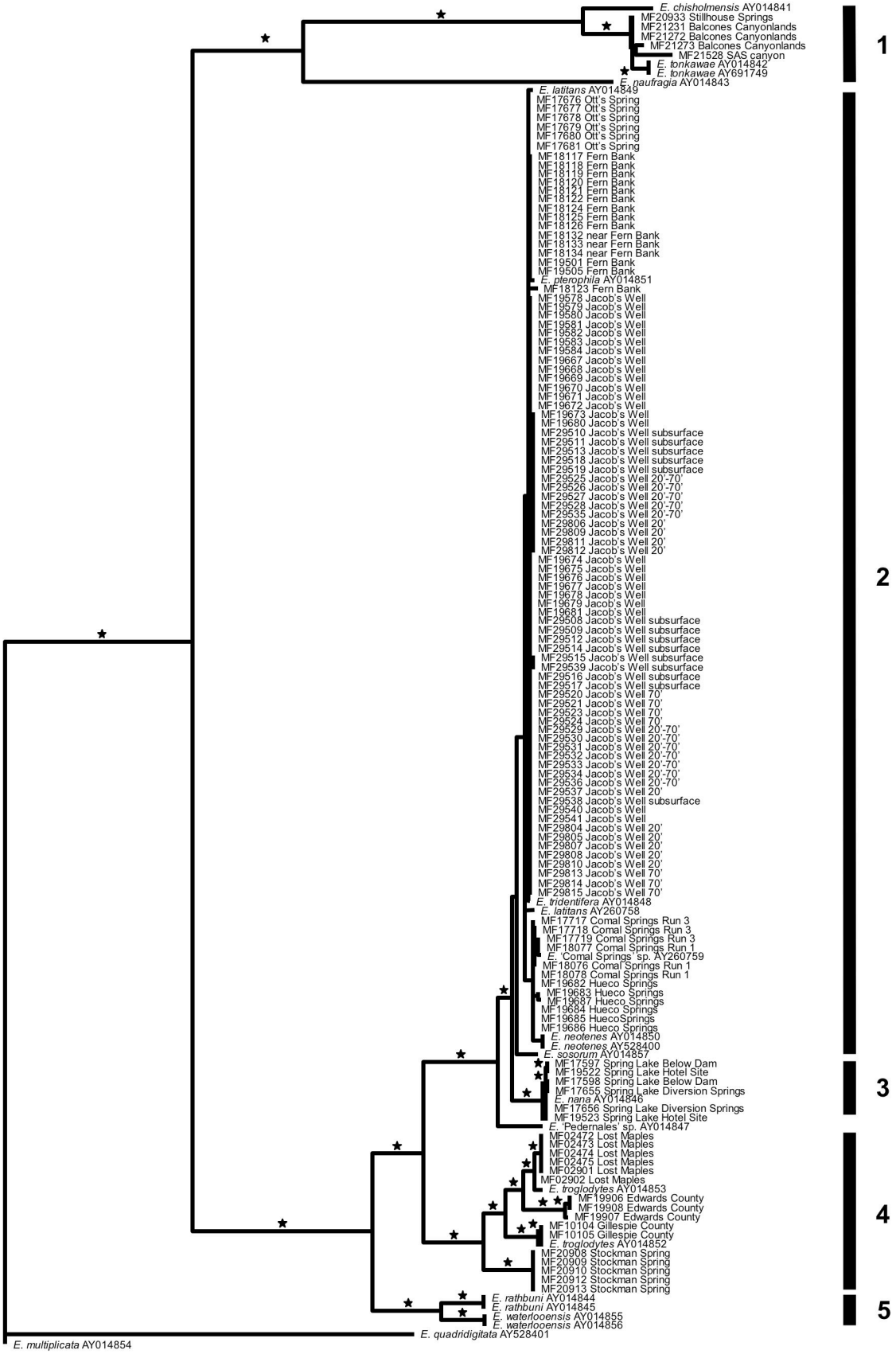
E. chisholmensis AY014841
MF20933 Stillhouse Springs
MF21231 Balcones Canyonlands
MF21272 Balcones Canyonlands
MF21273 Balcones Canyonlands
MF21528 SAS canyon
E. tonkawae AY014842
E. tonkawae AY691749
E. gaufragia AY014843
1
E. latitans AY014849
MF17676 Ott's Spring
MF17677 Ott's Spring
MF17678 Ott's Spring
MF17679 Ott's Spring
MF17680 Ott's Spring
MF17681 Ott's Spring
MF18117 Fern Bank
MF18118 Fern Bank
MF18119 Fern Bank
MF18120 Fern Bank
MF18121 Fern Bank
MF18122 Fern Bank
MF18124 Fern Bank
MF18125 Fern Bank
MF18126 Fern Bank
MF18132 near Fern Bank
MF18133 near Fern Bank
MF18134 near Fern Bank
MF19501 Fern Bank
MF19505 Fern Bank
E. pterophila AY014851
MF18123 Fern Bank
MF19578 Jacob's Well
MF19579 Jacob's Well
MF19580 Jacob's Well
MF19581 Jacob's Well
MF19582 Jacob's Well
MF19583 Jacob's Well
MF19584 Jacob's Well
MF19667 Jacob's Well
MF19668 Jacob's Well
MF19669 Jacob's Well
MF19670 Jacob's Well
MF19671 Jacob's Well
MF19672 Jacob's Well
MF19673 Jacob's Well
MF19680 Jacob's Well
MF29510 Jacob's Well subsurface
MF29511 Jacob's Well subsurface
MF29513 Jacob's Well subsurface
MF29518 Jacob's Well subsurface
MF29519 Jacob's Well subsurface
MF29525 Jacob's Well 20'-70'
MF29526 Jacob's Well 20'-70'
MF29527 Jacob's Well 20'-70'
MF29528 Jacob's Well 20'-70'
MF29535 Jacob's Well 20'-70'
MF29806 Jacob's Well 20'
MF29809 Jacob's Well 20'
MF29811 Jacob's Well 20'
MF29812 Jacob's Well 20'
MF19674 Jacob's Well
MF19675 Jacob's Well
MF19676 Jacob's Well
MF19677 Jacob's Well
MF19678 Jacob's Well
MF19679 Jacob's Well
MF19681 Jacob's Well
MF29508 Jacob's Well subsurface
MF29509 Jacob's Well subsurface
MF29512 Jacob's Well subsurface
MF29514 Jacob's Well subsurface
MF29515 Jacob's Well subsurface
MF29539 Jacob's Well subsurface
MF29516 Jacob's Well subsurface
MF29517 Jacob's Well subsurface
MF29520 Jacob's Well 70'
MF29521 Jacob's Well 70'
MF29523 Jacob's Well 70'
MF29524 Jacob's Well 70'
MF29529 Jacob's Well 20'-70'
MF29530 Jacob's Well 20'-70'
MF29531 Jacob's Well 20'-70'
MF29532 Jacob's Well 20'-70'
MF29533 Jacob's Well 20'-70'
MF29534 Jacob's Well 20'-70'
MF29536 Jacob's Well 20'-70'
MF29537 Jacob's Well 20'
MF29538 Jacob's Well subsurface
MF29540 Jacob's Well
MF29541 Jacob's Well
MF29804 Jacob's Well 20'
MF29805 Jacob's Well 20'
MF29807 Jacob's Well 20'
MF29808 Jacob's Well 20'
MF29810 Jacob's Well 20'
MF29813 Jacob's Well 70'
MF29814 Jacob's Well 70'
MF29815 Jacob's Well 70'
E. tridentifera AY014848
E. latitans AY260758
MF17717 Comal Springs Run 3
MF17718 Comal Springs Run 3
MF17719 Comal Springs Run 3
MF18077 Comal Springs Run 1
E. 'Comal Springs' sp. AY260759
MF18076 Comal Springs Run 1
MF18078 Comal Springs Run 1
MF19682 Hueco Springs
MF19683 Hueco Springs
MF19687 Hueco Springs
MF19684 Hueco Springs
MF19685 HuecoSprings
MF19686 Hueco Springs
E. neotenes AY014850
E. neotenes AY528400
2
E. sosorum AY014857
MF17597 Spring Lake Below Dam
MF19522 Spring Lake Hotel Site
MF17598 Spring Lake Below Dam
MF17655 Spring Lake Diversion Springs
E. nana AY014846
MF17656 Spring Lake Diversion Springs
MF19523 Spring Lake Hotel Site
E. 'Pedernales' sp. AY014847
3
MF02472 Lost Maples
MF02473 Lost Maples
MF02474 Lost Maples
MF02475 Lost Maples
MF02901 Lost Maples
MF02902 Lost Maples
E. troglodytes AY014853
MF19906 Edwards County
MF19908 Edwards County
MF19907 Edwards County
MF10104 Gillespie County
MF10105 Gillespie County
E. troglodytes AY014852
MF20908 Stockman Spring
MF20909 Stockman Spring
MF20910 Stockman Spring
MF20912 Stockman Spring
MF20913 Stockman Spring
4
E. rathbuni AY014844
E. rathbuni AY014845
E. waterlooensis AY014855
E. waterlooensis AY014856
5
E. quadridigitata AY528401
E. multiplicata AY014854
5 changes

Figure 1. Bayesian consensus phylogram of 43 unique mtDNA haplotypes (149 individuals) rooted with *Eurycea multiplicata*. Black vertical bars indicate five clades. Stars indicate posterior probabilities >87. Posterior probabilities for internal nodes in clade 2 are not shown (see Fig. 2).

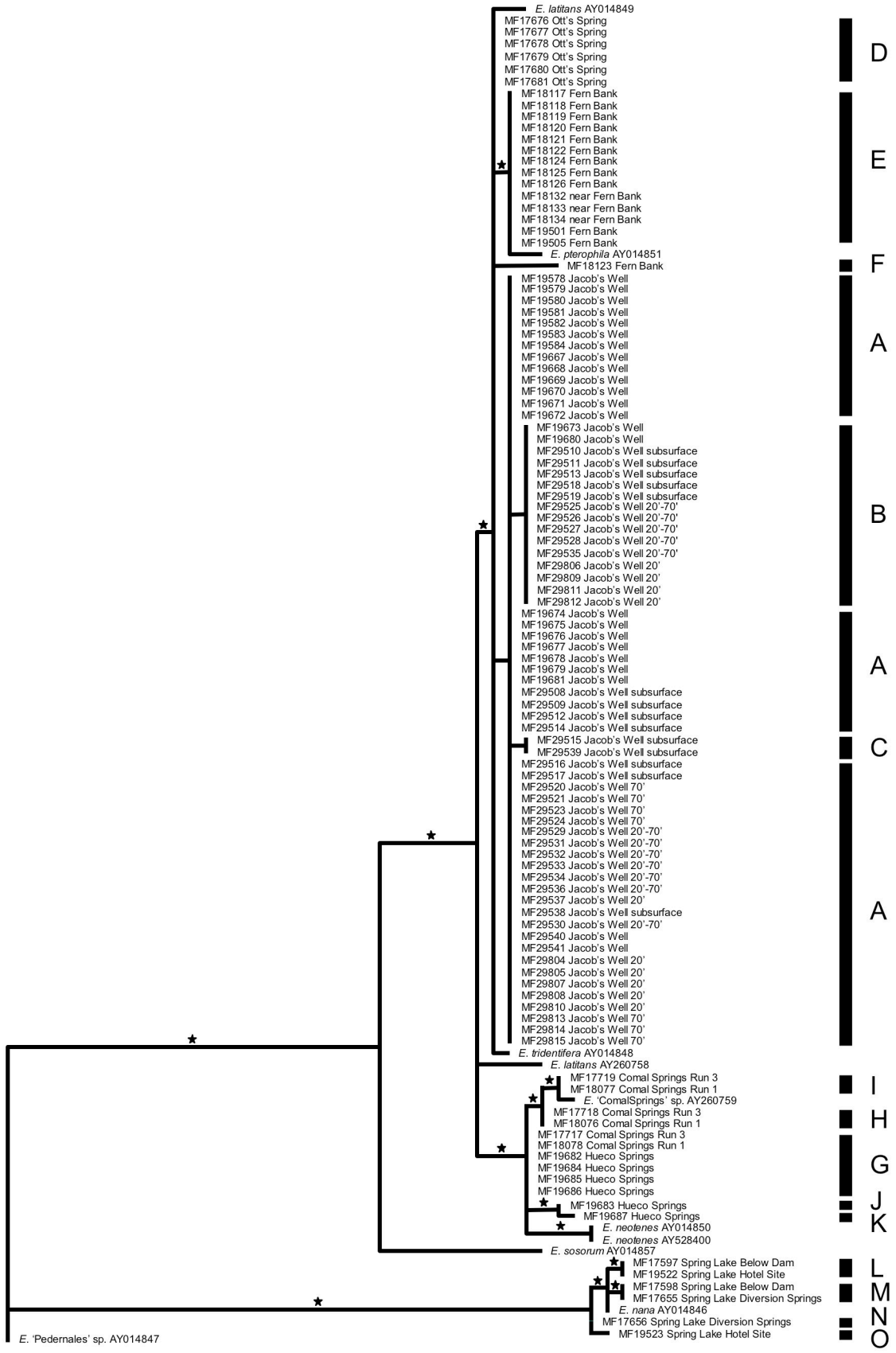
E. latitans AY014849
MF17676 Ott's Spring
MF17677 Ott's Spring
MF17678 Ott's Spring
MF17679 Ott's Spring
MF17680 Ott's Spring
MF17681 Ott's Spring
MF18117 Fern Bank
MF18118 Fern Bank
MF18119 Fern Bank
MF18120 Fern Bank
MF18121 Fern Bank
MF18122 Fern Bank
MF18124 Fern Bank
MF18125 Fern Bank
MF18126 Fern Bank
MF18132 near Fern Bank
MF18133 near Fern Bank
MF18134 near Fern Bank
MF19501 Fern Bank
MF19505 Fern Bank
E. pterophila AY014851
MF18123 Fern Bank
MF19578 Jacob's Well
MF19579 Jacob's Well
MF19580 Jacob's Well
MF19581 Jacob's Well
MF19582 Jacob's Well
MF19583 Jacob's Well
MF19584 Jacob's Well
MF19667 Jacob's Well
MF19668 Jacob's Well
MF19669 Jacob's Well
MF19670 Jacob's Well
MF19671 Jacob's Well
MF19672 Jacob's Well
MF19673 Jacob's Well
MF19680 Jacob's Well
MF29510 Jacob's Well subsurface
MF29511 Jacob's Well subsurface
MF29513 Jacob's Well subsurface
MF29518 Jacob's Well subsurface
MF29519 Jacob's Well subsurface
MF29525 Jacob's Well 20'-70'
MF29526 Jacob's Well 20'-70'
MF29527 Jacob's Well 20'-70'
MF29528 Jacob's Well 20'-70'
MF29535 Jacob's Well 20'-70'
MF29806 Jacob's Well 20'
MF29809 Jacob's Well 20'
MF29811 Jacob's Well 20'
MF29812 Jacob's Well 20'
MF19674 Jacob's Well
MF19675 Jacob's Well
MF19676 Jacob's Well
MF19677 Jacob's Well
MF19678 Jacob's Well
MF19679 Jacob's Well
MF19681 Jacob's Well
MF29508 Jacob's Well subsurface
MF29509 Jacob's Well subsurface
MF29512 Jacob's Well subsurface
MF29514 Jacob's Well subsurface
MF29515 Jacob's Well subsurface
MF29539 Jacob's Well subsurface
MF29516 Jacob's Well subsurface
MF29517 Jacob's Well subsurface
MF29520 Jacob's Well 70'
MF29521 Jacob's Well 70'
MF29523 Jacob's Well 70'
MF29524 Jacob's Well 70'
MF29529 Jacob's Well 20'-70'
MF29531 Jacob's Well 20'-70'
MF29532 Jacob's Well 20'-70'
MF29533 Jacob's Well 20'-70'
MF29534 Jacob's Well 20'-70'
MF29536 Jacob's Well 20'-70'
MF29537 Jacob's Well 20'
MF29538 Jacob's Well subsurface
MF29530 Jacob's Well 20'-70'
MF29540 Jacob's Well
MF29541 Jacob's Well
MF29804 Jacob's Well 20'
MF29805 Jacob's Well 20'
MF29807 Jacob's Well 20'
MF29808 Jacob's Well 20'
MF29810 Jacob's Well 20'
MF29813 Jacob's Well 70'
MF29814 Jacob's Well 70'
MF29815 Jacob's Well 70'
E. tridentifera AY014848
E. latitans AY260758
MF17719 Comal Springs Run 3
MF18077 Comal Springs Run 1
E. 'ComalSprings' sp. AY260759
MF17718 Comal Springs Run 3
MF18076 Comal Springs Run 1
MF17717 Comal Springs Run 3
MF18078 Comal Springs Run 1
MF19682 Hueco Springs
MF19684 Hueco Springs
MF19685 Hueco Springs
MF19686 Hueco Springs
MF19683 Hueco Springs
MF19687 Hueco Springs
E. neotenes AY014850
E. neotenes AY528400
E. sosorum AY014857
MF17597 Spring Lake Below Dam
MF19522 Spring Lake Hotel Site
MF17598 Spring Lake Below Dam
MF17655 Spring Lake Diversion Springs
E. nana AY014846
MF17656 Spring Lake Diversion Springs
MF19523 Spring Lake Hotel Site
E. 'Pedernales' sp. AY014847
0.5 changes
D
E
F
A
B
A
C
A
I
H
G
J
K
L
M
N
O

Figure 2. Bayesian consensus phylogram of 24 unique mtDNA haplotypes (116 individuals) rooted with *Eurycea* 'Pedernales' sp. Black vertical bars indicate haplotypes generated in this study. Stars indicate posterior probabilities >92.

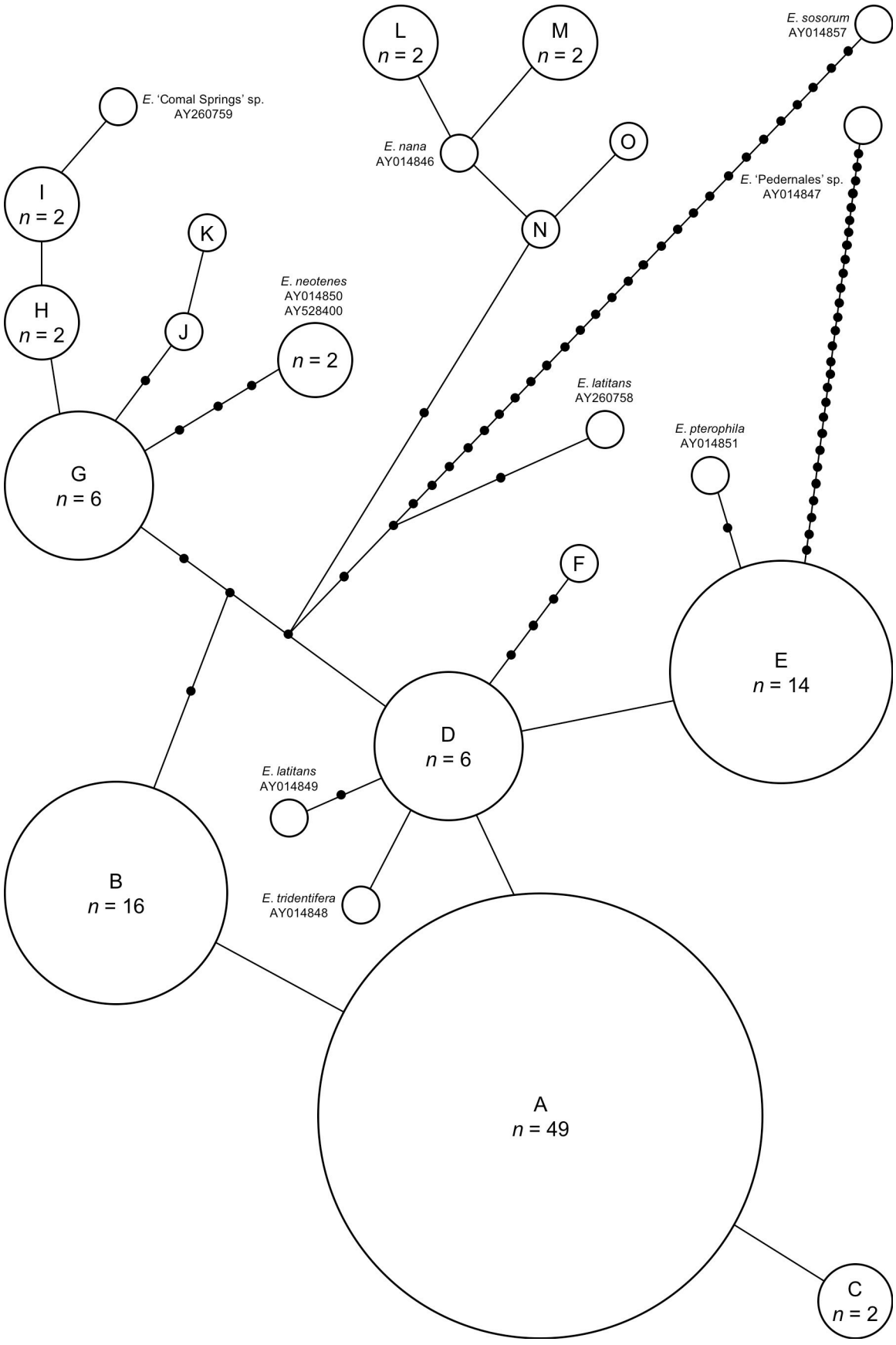
L
n = 2
M
n = 2
E. sosorum
AY014857
E. 'Comal Springs' sp.
AY260759
E. nana
AY014846
O
E. 'Pedernales' sp.
AY014847
I
n = 2
K
N
E. neotenes
AY014850
AY528400
H
n = 2
J
n = 2
E. latitans
AY260758
E. pterophila
AY014851
G
n = 6
F
E
n = 14
D
n = 6
E. latitans
AY014849
B
n = 16
E. tridentifera
AY014848
A
n = 49
C
n = 2

Figure 3. Statistical parismony network of 24 unique mtDNA haplotypes in 116 *Eurycea* (same as those in Fig. 2). Circle size is proportional to number of individuals. Haplotype names (e.g., A or *E. nana* AY014846) and sample sizes are shown (n = 1 where sample size is not indicated). Each line represents a single mutation; small filled circles represent nonsampled or extinct haplotypes. Nineteen of the mutations separating *E. sosorum* from the other haplotypes are gaps from the 5' end of the alignment; missing data were treated as a 5th state to reconstruct this network.

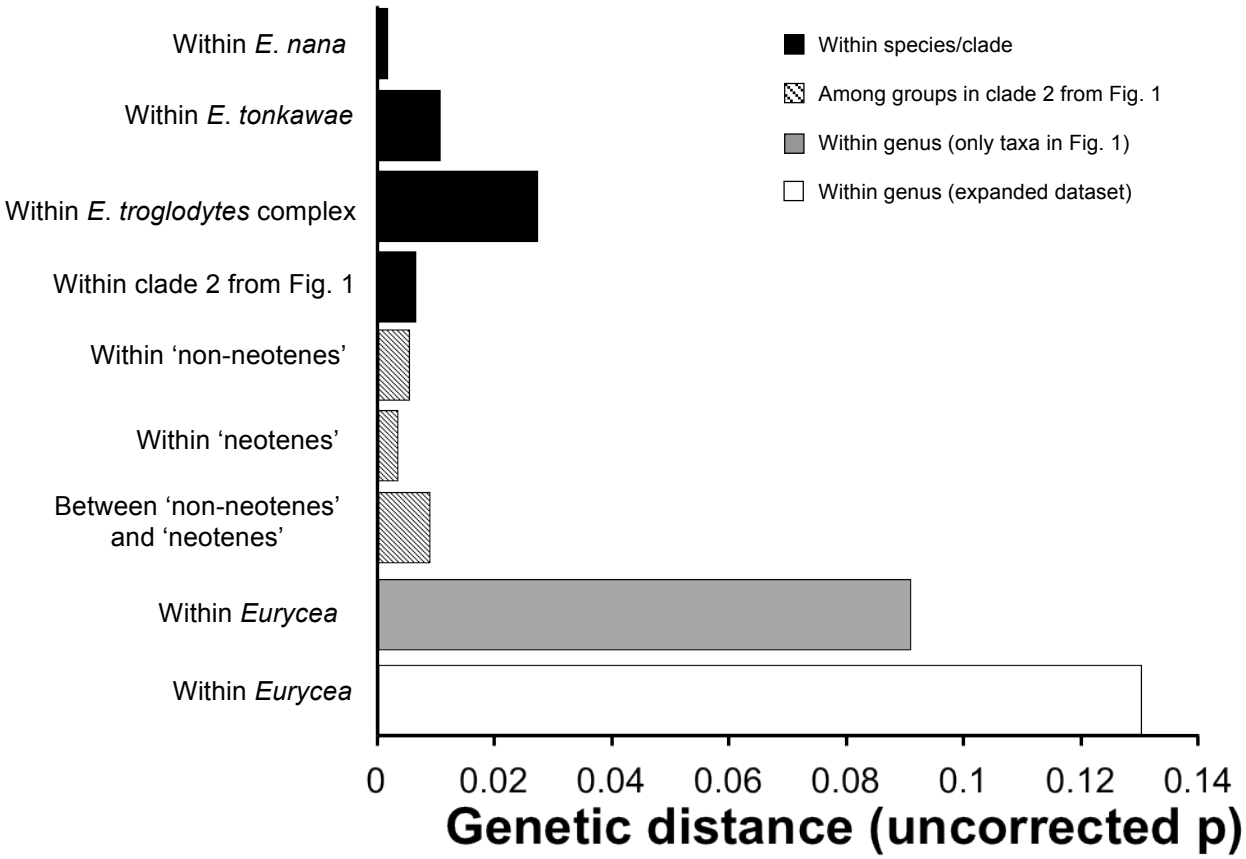
Within species/clade
Among groups in clade 2 from Fig. 1
Within genus (only taxa in Fig. 1)
Within genus (expanded dataset)
Within E. nana
Within E. tonkawae
Within E. troglodytes complex
Within clade 2 from Fig. 1
Within 'non-neotenes'
Within 'neotenes'
Between 'non-neotenes' and 'neotenes'
Within Eurycea
Within Eurycea
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
Genetic distance (uncorrected p)

Figure 4. Genetic distances (uncorrected p) for multiple taxonomic/phylogenetic levels in the genus *Eurycea*. Each bar represents the average of all possible comparisons between unique haplotypes. The expanded dataset (n = 241) included GenBank sequences from *E. bislineata*, *E. guttolineata*, *E. longicauda*. *E. lucifuga*, *E. spelaeus*, and *E. tynerensis*. 'Neotenes' includes haplotypes G, H, I, J, K, *E.* 'Comal Springs' sp., and *E. neotenes*. 'Non-neotenes' includes haplotypes, A, B, C, D, E, F, *E. latitans*, *E. pterophila*, *E. sosorum*, and *E. tridentifera*.

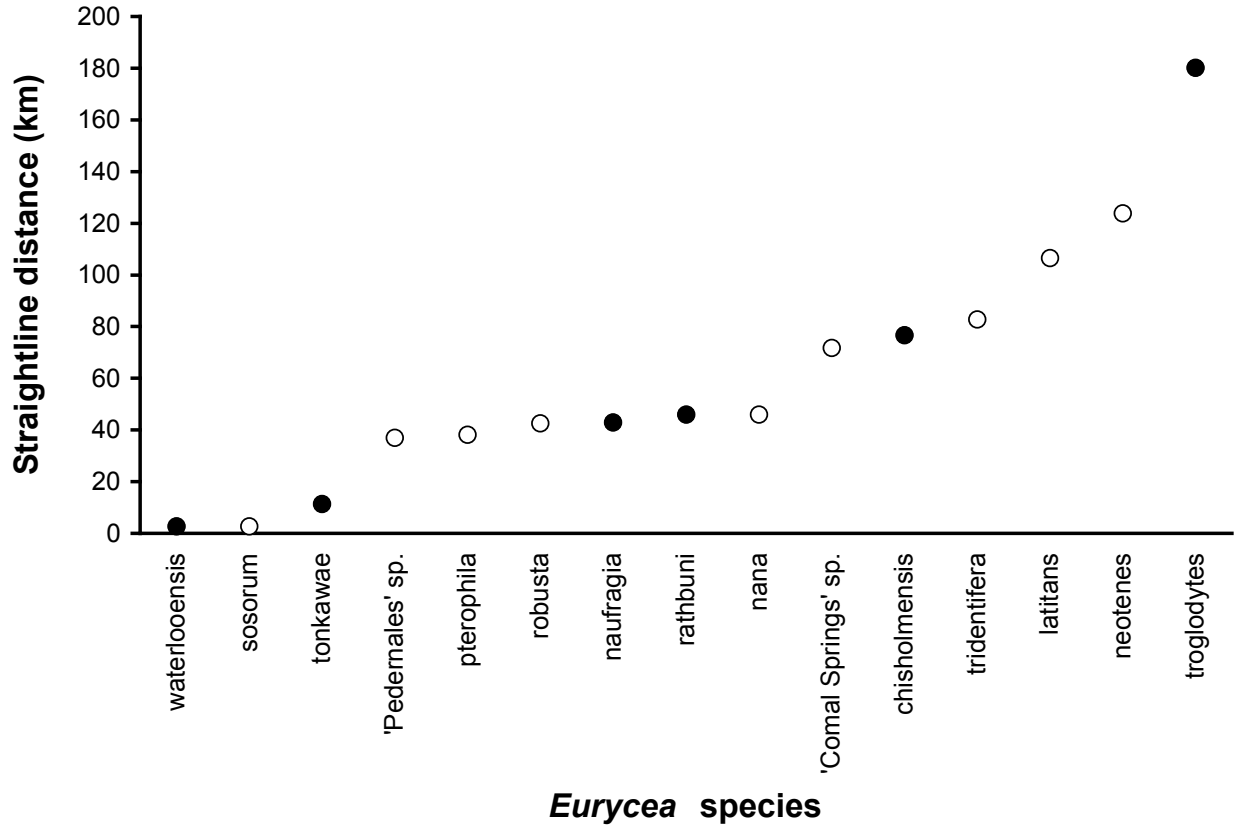

Figure 5. Straightline distance (in km) from the Texas State Capitol building in Austin, Texas, to the type localities of central Texas *Eurycea* species. Open circles represent species which were recovered in clade 2 from Fig. 1.

Appendix A. MF number (Michael R. J. Forstner Frozen Tissue catalog identification number), collector number, species (as identified in the field), snout-vent length (SVL, in mm), tail length (in mm), total length (in mm), date collected, county and site of collection, and mtDNA haplotype for individuals used in this study.

MF Number	Collector Number	species	SVL	Tail Length	Total Length	Date Collected	County	Site	Haplotype
2472		sp.					Bandera	Lost Maples Natural Area	T
2473		sp.					Bandera	Lost Maples Natural Area	T
2474		sp.					Bandera	Lost Maples Natural Area	T
2475		sp.					Bandera	Lost Maples Natural Area	T
2901		sp.					Bandera	Lost Maples Natural Area	T
2902		sp.					Bandera	Lost Maples Natural Area	U
10104		sp.					Gillespie	1.36 rd mi S jct White Oak Rd & Zenner-Alherns Rd on Zenner-Alherns Rd	X
10105		sp.					Gillespie	1.36 rd mi S jct White Oak Rd & Zenner-Alherns Rd on Zenner-Alherns Rd	Z
17597		nana					Hays	Spring Lake Below Dam	L
17598		nana					Hays	Spring Lake Below Dam	M
17655		nana					Hays	Spring Lake Diversion Springs	M
17656		nana					Hays	Spring Lake Diversion Springs	N
17676		sp.					Comal	Ott's Spring	D
17677		sp.					Comal	Ott's Spring	D
17678		sp.					Comal	Ott's Spring	D
17679		sp.					Comal	Ott's Spring	D
17680		sp.					Comal	Ott's Spring	D
17681		sp.					Comal	Ott's Spring	D
17717		sp.					Comal	Comal Springs Run 3	G
17718		sp.					Comal	Comal Springs Run 3	H
17719		sp.					Comal	Comal Springs Run 3	I
18076		sp.					Comal	Comal Springs Run 1	H
18077		sp.					Comal	Comal Springs Run 1	I
18078		sp.					Comal	Comal Springs Run 1	G
18117		sp.					Hays	Fern Bank Springs	E
18118		sp.					Hays	Fern Bank Springs	E
18119		sp.					Hays	Fern Bank Springs	E
18120		sp.					Hays	Fern Bank Springs	E
18121		sp.					Hays	Fern Bank Springs	E
18122		sp.					Hays	Fern Bank Springs	E
18123		sp.					Hays	Fern Bank Springs	F
18124		sp.					Hays	Fern Bank Springs	E
18125		sp.					Hays	Fern Bank Springs	E
18126		sp.					Hays	Fern Bank Springs	E
18132		sp.					Hays	near Fern Bank Springs	E
18133		sp.					Hays	near Fern Bank Springs	E
18134		sp.					Hays	near Fern Bank Springs	E
19501		sp.					Hays	Fern Bank Springs	E
19505		sp.					Hays	Fern Bank Springs	E
19522		nana					Hays	Spring Lake Hotel site	L
19523		nana					Hays	Spring Lake Hotel site	O
19578		sp.					Hays	Jacob's Well	A
19579		sp.					Hays	Jacob's Well	A
19580		sp.					Hays	Jacob's Well	A
19581		sp.					Hays	Jacob's Well	A
19582		sp.					Hays	Jacob's Well	A
19583		sp.					Hays	Jacob's Well	A
19584		sp.					Hays	Jacob's Well	A
19667		sp.					Hays	Jacob's Well	A
19668		sp.					Hays	Jacob's Well	A
19669		sp.					Hays	Jacob's Well	A
19670		sp.					Hays	Jacob's Well	A

MF Number	Collector Number	species	SVL	Tail Length	Total Length	Date Collected	County	Site	Haplotype
19671		sp.					Hays	Jacob's Well	A
19672		sp.					Hays	Jacob's Well	A
19673		sp.					Hays	Jacob's Well	B
19674		sp.					Hays	Jacob's Well	A
19675		sp.					Hays	Jacob's Well	A
19676		sp.					Hays	Jacob's Well	A
19677		sp.					Hays	Jacob's Well	A
19678		sp.					Hays	Jacob's Well	A
19679		sp.					Hays	Jacob's Well	A
19680		sp.					Hays	Jacob's Well	B
19681		sp.					Hays	Jacob's Well	A
19682		sp.					Comal	Hueco Springs	G
19683		sp.					Comal	Hueco Springs	J
19684		sp.					Comal	Hueco Springs	G
19685		sp.					Comal	Hueco Springs	G
19686		sp.					Comal	Hueco Springs	G
19687		sp.					Comal	Hueco Springs	K
19906		troglodytes complex					Edwards	250 m W of RR335, about 8.5 rd km S of jct RR335 & TX Hwy 41	V
19907		troglodytes complex					Edwards	250 m W of RR335, about 8.5 rd km S of jct RR335 & TX Hwy 41	W
19908		troglodytes complex					Edwards	250 m W of RR335, about 8.5 rd km S of jct RR335 & TX Hwy 41	V
20908		sp.					Kerr	Stockman Spring	Y
20909		sp.					Kerr	Stockman Spring	Y
20910		sp.					Kerr	Stockman Spring	Y
20912		sp.					Kerr	Stockman Spring	Y
20913		sp.					Kerr	Stockman Spring	Y
20933		tonkawae					Travis	Stillhouse Springs	P
21231		tonkawae					Travis	Balcones Canyonlands, mainstem above Tributary 7	Q
21272		tonkawae					Travis	Balcones Canyonlands, Tributary 5	Q
21273		tonkawae					Travis	Balcones Canyonlands, Tributary 5	R
21528		tonkawae					Travis	SAS canyon	S
29508	Zara-5563	sp.			8.2	5/21/10	Hays	Jacob's Well subsurface	A
29509	Zara-5564	sp.	8.5	6.4	14.9	5/21/10	Hays	Jacob's Well subsurface	A
29510	Zara-5564	sp.	13	8.8	21.8	5/21/10	Hays	Jacob's Well subsurface	B
29511	Zara-5564	sp.	10.6	6	16.6	5/21/10	Hays	Jacob's Well subsurface	B
29512	Zara-5564	sp.	9.7	4.6	14.3	5/21/10	Hays	Jacob's Well subsurface	A
29513	Zara-5564	sp.	8.8	4.6	13.4	5/21/10	Hays	Jacob's Well subsurface	B
29514	Zara-5564	sp.	9.2	5.3	14.5	5/21/10	Hays	Jacob's Well subsurface	A
29515	Zara-5564	sp.	12.5	6.6	19.1	5/21/10	Hays	Jacob's Well subsurface	C
29516	Zara-5564	sp.	12.1	7.2	19.3	5/21/10	Hays	Jacob's Well subsurface	A
29517	Zara-5564	sp.	9.2	5.1	14.3	5/21/10	Hays	Jacob's Well subsurface	A
29518	Zara-5564	sp.	11.6	5.8	17.4	5/21/10	Hays	Jacob's Well subsurface	B
29519	Zara-5564	sp.	12.6	6.9	19.5	5/21/10	Hays	Jacob's Well subsurface	B
29520	Zara-5565	sp.	9.1	5	14.1	5/14/10	Hays	Jacob's Well 70'	A
29521	Zara-5565	sp.	8.4	4.7	13.1	5/14/10	Hays	Jacob's Well 70'	A
29522	Zara-5565	sp.	9.4	5	14.4	5/14/10	Hays	Jacob's Well 70'	n/a
29523	Zara-5565	sp.	9.3	6	15.3	5/14/10	Hays	Jacob's Well 70'	A
29524	Zara-5565	sp.	9.4	5.5	14.9	5/14/10	Hays	Jacob's Well 70'	A
29525	Zara-5566	sp.	13	6.6	19.6	6/10/10	Hays	Jacob's Well 20'-70'	B
29526	Zara-5566	sp.	9	4.1	13.1	6/10/10	Hays	Jacob's Well 20'-70'	B
29527	Zara-5566	sp.	9.8	4.7	14.5	6/10/10	Hays	Jacob's Well 20'-70'	B
29528	Zara-5566	sp.	9.2	3.4	12.6	6/10/10	Hays	Jacob's Well 20'-70'	B
29529	Zara-5566	sp.	10.1	5.3	15.4	6/10/10	Hays	Jacob's Well 20'-70'	A
29530	Zara-5566	sp.	9.8	4.9	14.7	6/10/10	Hays	Jacob's Well 20'-70'	A
29531	Zara-5566	sp.	8.8	4.5	13.3	6/10/10	Hays	Jacob's Well 20'-70'	A
29532	Zara-5566	sp.	8.9	4.7	13.6	6/10/10	Hays	Jacob's Well 20'-70'	A
29533	Zara-5566	sp.	9.5	6.6	16.1	6/10/10	Hays	Jacob's Well 20'-70'	A

MF Number	Collector Number	species	SVL	Tail Length	Total Length	Date Collected	County	Site	Haplotype
29534	Zara-5566	sp.	9	5.1	14.1	6/10/10	Hays	Jacob's Well 20'-70'	A
29535	Zara-5566	sp.	8.8	5.2	14	6/10/10	Hays	Jacob's Well 20'-70'	B
29536	Zara-5566	sp.	10.7	6.3	17	6/10/10	Hays	Jacob's Well 20'-70'	A
29537	Zara-5569	sp.				6/5/10	Hays	Jacob's Well 20'	A
29538	Zara-5570	sp.				8/13/09	Hays	Jacob's Well subsurface	A
29539	Zara-5571	sp.				5/28/09	Hays	Jacob's Well subsurface	C
29540	Zara-5576	sp.				5/14/10	Hays	Jacob's Well	A
29541	Zara-5577	sp.				5/14/10	Hays	Jacob's Well	A
29804	Zara-5934	sp.	7.3	3.1	10.4	7/3/10	Hays	Jacob's Well 20'	A
29805	Zara-5934	sp.	8.1	6.2	14.3	7/3/10	Hays	Jacob's Well 20'	A
29806	Zara-5934	sp.	10.9	5.8	16.7	7/3/10	Hays	Jacob's Well 20'	B
29807	Zara-5934	sp.	9.7	4.7	14.4	7/3/10	Hays	Jacob's Well 20'	A
29808	Zara-5934	sp.	9.7	5.4	15.1	7/3/10	Hays	Jacob's Well 20'	A
29809	Zara-5934	sp.	13.8	9.5	23.4	7/3/10	Hays	Jacob's Well 20'	B
29810	Zara-5934	sp.	18.6	6.9	25.5	7/3/10	Hays	Jacob's Well 20'	A
29811	Zara-5934	sp.	17.6	12.5	30.1	7/3/10	Hays	Jacob's Well 20'	B
29812	Zara-5935	sp.	31.3	24.4	55.7	7/3/10	Hays	Jacob's Well 20'	B
29813	Zara-5936	sp.	8.6	4.1	12.7	8/4/10	Hays	Jacob's Well 70'	A
29814	Zara-5936	sp.	10	4.8	14.8	8/4/10	Hays	Jacob's Well 70'	A
29815	Zara-5936	sp.	9.8	5.3	15.1	8/4/10	Hays	Jacob's Well 70'	A