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RESEARCH ARTICLE

Geographic and genetic isolation in spring-associated *Eurycea* salamanders endemic to the Edwards Plateau region of Texas

Lauren K. Lucas · Zachariah Gompert · James R. Ott · Chris C. Nice

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Abstract Populations of neotenic, spring-associated salamanders of the genus *Eurycea* occupy discontinuous sites throughout the Edwards Plateau of central Texas and many warrant conservation attention. Here we used DNA sequence data from a nuclear (*rag1*) and a mitochondrial (*ND4*) gene to determine (1) the extent of genetic isolation among seven Edwards Plateau *Eurycea* populations and (2) the relationship between genetic distance and both geographic distance and hydrogeological features. Coalescent-based methods detected little gene flow among the sampled *Eurycea* populations, and we were unable to reject a model of complete isolation for any pair of populations. These findings were consistent with the relatively high genetic distances we detected among the sampled *Eurycea* populations (pairwise ϕ_{ST} ranged from 0.249 to 0.924). We detected a positive correlation between genetic distance and geographic distance, which is consistent with a pattern of isolation by distance. However, while controlling for geographic distance, we did not detect a positive relationship between genetic distance and aquifer or river distance. Thus, we found no evidence that aquifers and/or rivers serve as dispersal corridors among isolated *Eurycea* populations. Based on these results, we have no evidence that re-colonization of spring sites by migrant salamanders following local extirpation would be likely. Our findings indicate that spring-associated *Eurycea* salamander populations occupying the Edwards Plateau region are genetically isolated, and that each of these populations should be considered a distinct management unit.

Keywords *Eurycea* · Migration rate · Coalescent · Endemism · Isolation by distance · Edwards Plateau

L. K. Lucas · J. R. Ott · C. C. Nice (✉)
Department of Biology, Population and Conservation Biology Program, Texas State University, 601 University Dr., San Marcos, TX 78666, USA
e-mail: ccnice@txstate.edu

Z. Gompert
Department of Botany, University of Wyoming, 1000 E. University Ave., Laramie, WY 82071, USA

Introduction

Understanding the extent of migration (gene flow) among isolated populations is relevant for conservation and management efforts for two primary reasons. First, migration directly affects ecological and evolutionary processes (Hanski and Gilpin 1997; Whitlock and McCauley 1999). Migration can maintain allelic variation within populations and contribute to the spread of adaptive alleles among populations (Slatkin 1987; Frankham et al. 2002; Furmankiewicz and Altringham 2007). However, migration can also result in outbreeding depression and in some instances retard local adaptation (Slatkin 1987; Whitlock and McCauley 1999; Wang et al. 2007; Roberge et al. 2008). Second, estimates of migration rates and knowledge of the conduits by which migration occurs can inform efforts to delineate conservation or management units. For example, the amount of migration among populations determines the extent that ecological and evolutionary processes in a population influence processes in other populations (Moritz 1999; Whitlock and McCauley 1999). Herein, we examine geographic patterns of genetic variation to estimate migration rates and identify possible conduits for migration in a group of salamanders in the genus *Eurycea* (Plethodontidae: Hemidactyliini) found in

the Edwards Plateau region of central Texas, commonly known as the Hill Country. This study will inform conservation practices and aid efforts to delineate management units within this group.

Edwards Plateau *Eurycea* are neotenic, and thus, restricted to aquatic habitats their entire lives. Two general *Eurycea* lineages occur in the Edwards Plateau region: members of the *Typhlomolge* clade, which are strictly found in caves, and members of the *Blepsimolge* clade, which are found in both surface springs and caves. This study is concerned solely with spring-associated *Eurycea* of the *Blepsimolge* clade. Spring-associated *Eurycea* are dependent on the stable conditions of the spring systems, such as near constant water temperature, pH, flow, and dissolved oxygen, as well as minimal substrate embeddedness (Tupa and Davis 1976; Nelson 1993). Springs in the Edwards Plateau emanate from the porous limestone of the Edwards, Trinity, and Edwards–Trinity Aquifers, the former being one of the most productive reservoirs of potable ground water in the United States, as well as one of the most species-rich aquifers in the world (Longley 1981). The Edwards Plateau has been dissected and eroded forming numerous springs and caves (Abbott and Woodruff 1986; Barker et al. 1994). Erosion and stream piracy have created isolated habitats that are inhabited by *Eurycea*, as well as a variety of other endemic aquatic organisms (Brune 1981; Telfair 1999), many of which are also federally listed within the USA as threatened or endangered species due to their restricted ranges and threats to their habitat (Brune 1981). This conservation concern is not trivial, as the human population of the Edwards Plateau is growing rapidly, which will place continuing demands on the region's aquatic resources (Brune 1981). This problem may be exacerbated by local climate change (Loáiciga et al. 2000; Chen et al. 2001).

While *Eurycea* (*Blepsimolge* clade) populations associated with different Edwards Plateau springs may experience nearly complete geographical isolation, these populations may exchange migrants. Specifically, migration among populations may occur via subterranean connections within aquifers or by surface connections within river drainage systems, or both. If spring-associated *Eurycea* populations do not exchange migrants, then each population may be considered to represent an independent evolutionary unit, and perhaps a separate conservation unit. This level of isolation may result in an increased probability of local and/or regional extinction of Edwards Plateau *Eurycea*. Conversely, if substantial migration occurs among spring-associated *Eurycea* populations then these populations could be considered a single meta-population and managed accordingly. This second scenario would make maintenance of potential conduits for gene flow (i.e., rivers and/or aquifers) a greater conservation priority (Segelbacher et al. 2003).

Here we examine geographic patterns of population genetic variation to determine if spring-associated Edwards Plateau *Eurycea* (*Blepsimolge* clade) populations are connected by migration. Specifically, we address two questions: (1) to what extent does migration occur among the sampled *Eurycea* populations? (2) how do geographic distance and hydrogeological features (i.e., rivers and/or aquifers) affect genetic isolation among Edwards Plateau *Eurycea* populations? Addressing these questions will help focus and inform conservation efforts for Edwards Plateau *Eurycea* populations.

Methods

Population sampling

DNA was sampled from 254 *Eurycea* collected from seven springs in the Edwards Plateau during 2004 and 2005 (Table 1; Fig. 1). Four of the seven sampled populations have clear species designations: *Eurycea nana* in San Marcos Springs, *Eurycea pterophila* in Jacob's Well and Fern Bank, and *Eurycea neotenes* in Comal Springs (Chippindale et al. 2000; Hillis et al. 2001) (Table 1). The taxonomic identification for the remaining three populations is more ambiguous, but based on geographic locality the Hueco Springs population should be considered *E. neotenes*, while Ott's Spring and Devil's Backbone should be classified as *E. pterophila* (although *E. pterophila* was previously described as occurring solely in the Blanco River drainage) (Chippindale et al. 2000). Recent efforts to delineate species boundaries within Edwards Plateau *Eurycea* have relied primarily on molecular data, although morphological characters have also been used (Chippindale et al. 2000; Hillis et al. 2001). Based on mitochondrial DNA (mtDNA), populations found north and south of the Colorado River exhibit 17.5% mtDNA sequence divergence; however, little molecular divergence has been detected among a subgroup of species of *Blepsimolge* south of the Colorado River (i.e., the "southeastern" clade), which includes the *Blepsimolge* species examined in this manuscript (Hillis et al. 2001). The low level of sequence divergence among the nominal species of the "southeastern" *Blepsimolge* clade (on the order of 2% maximum mtDNA sequence divergence, Hillis et al. 2001) suggests that this group represents a fairly recent radiation. Therefore, reproductive isolation may not be complete among these species, and interspecific gene flow may occur. Because of the possibility

Table 1 Aquifer, river and geographic location of sampled populations and haplotypes (number of individuals per haplotype) in each population

Population	Nominal taxon	Aquifer	River	Latitude/longitude	*n*	*ND4* (*n*)	*rag1* (*n*)
Jacob's Well	*E. pterophila*	Trinity	Blanco	30°2′3″ N 98°7′20″ W	22	nA (20), nB (2)	rC (1), rD (11), rE (8)
Ott's Spring	*E. pterophila* (?)	Trinity	Guadalupe	29°55′22″ N 98°9′3″ W	18	nA (1), nD (11), nF (6)	rD (9), rE (7)
Devil's Backbone	*E. pterophila* (?)	Trinity	Guadalupe	29°55′31″ N 98°9′18″ W	18	nA (9), nM (2), nL (7)	rD (25), rE (3)
Fern Bank	*E. pterophila*	Trinity	Blanco	29°59′38″ N 97°59′45″ W	37	nE (32), nG (4), nH (1)	rC (27), rD (2), rE (6), rG (7)
Comal Springs	*E. neotenes*	Edwards	Guadalupe	29°42′48″ N 98°8′7″ W	43	nJ (34), nK (9)	rC (61), rG (1)
Hueco Springs	*E. neotenes* (?)	Edwards	Guadalupe	29°45′56″ N 98°8′31″ W	13	nN (13)	rC (8), rD (14), rG (2)
San Marcos Springs	*E. nana*	Edwards	Blanco	29°53′33″ N 97°56′1″ W	103	nC (92), nI (11)	rA (51), rB (86), rF (1)

Taxonomic designations follow Chippindale et al. (2000); ambiguous taxonomic designations are denoted with a question mark (?)

of gene flow among these nominal *Eurycea* species, we adopt a population-level approach to understanding patterns of gene flow and treat all seven sampled locations as populations within a series. Population-level information is particularly important given that several spring-associated *Eurycea* populations in the Edwards Plateau have received federal or state conservation status (USFWS 1980).

Each sampled spring was associated with the origin of a first-order spring-fed intermittent stream within either the Blanco or Guadalupe River systems, and each spring emanated from either the Edwards or Trinity Aquifers (Table 1; Fig. 1). The San Marcos Springs population of the federally threatened *E. nana* was collected under permits (US Fish and Wildlife Service (TE676811-0) and Texas Parks and Wildlife (SPR-0390-045)). All other populations were collected with permission from landowners. Tissue samples were collected from each salamander by removing approximately 5 mm of tail using sterile equipment. Individuals were either released after tail clippings were taken or transported to the San Marcos National Fish Hatchery and Technology Center to augment existing captive populations. Tissue samples were stored in 95% ethanol at −80°C until DNA was extracted.

DNA extraction and sequencing

Total genomic DNA was extracted using commercially available kits (Promega Wizard SV Genomic DNA Purification System and Gentra Systems Puregene DNA Purification Kit). We sequenced an 817 bp fragment of a mtDNA gene region consisting of the 3′ portion of *NADH* subunit 4, *tRNA-His*, *tRNA-Ser* and the 5′ portion of *tRNA-Leu* (referred to as *ND4*) using the primers ND4F and LEUR (Arevalo et al. 1994). We also sequenced a 721 bp portion of the nuclear gene *recombination activating gene 1* (*rag1*) using primers designed for this study: RAG1-F (5′ CAA CTG GAC GGC AGA TTT TC 3′), RAG1-IF2 (5′ TTG AAC TTG GGG GCA TAC TC 3′), and RAG1-R (5′ TCC AGA TTC GTT CCC TTC AC 3′). Fluorescently labeled dideoxy terminators were used for single stranded sequencing reactions for both *ND4* and *rag1* according to Beckman Coulter, Inc. (Fullerton, CA) specifications. Labeled amplicons were separated and visualized using a capillary DNA sequencer (CEQ model 8800). Sequences were aligned using the program SEQUENCHER 4.5. Because heterozygosity was never complex (i.e., never involved more than one site), the allelic composition of each *rag1* heterozygote was easily determined by eye to produce fully

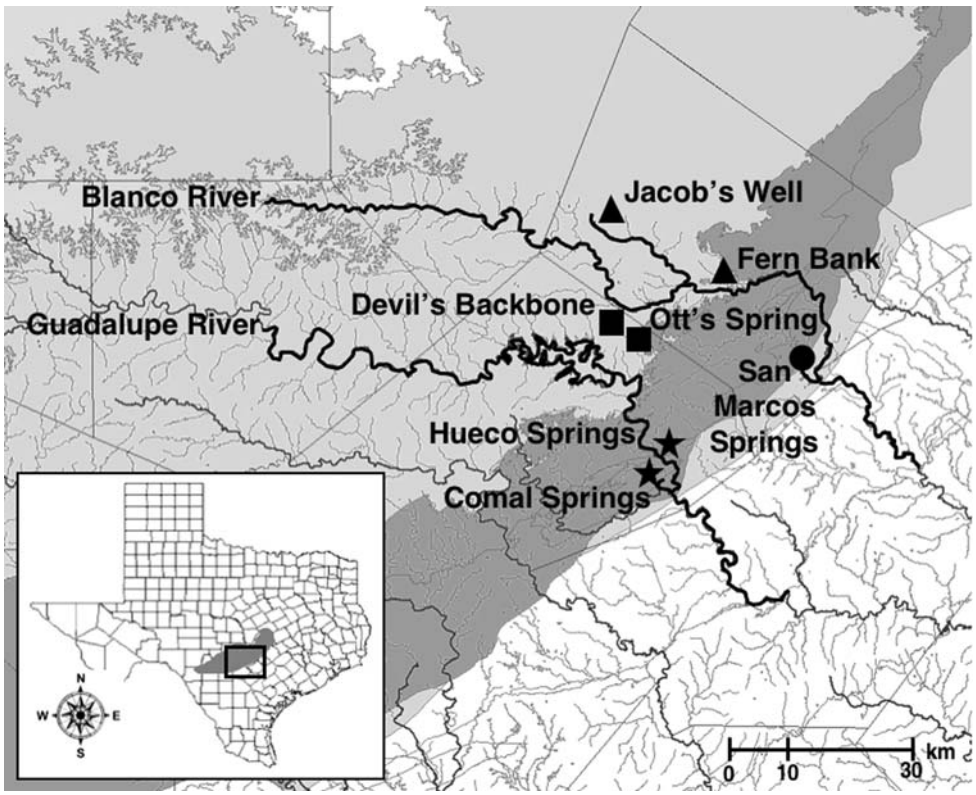

Fig. 1 Geographical distribution of neotenic, spring-associated *Eurycea* in Texas and sampling localities. The shaded area on the Texas map represents the distribution of spring-associated *Eurycea* in Texas. The light gray area on the fine-scale map represents the Trinity Aquifer, and dark gray represents the Edwards Aquifer. Triangles indicate populations found on the Trinity Aquifer and the Blanco River system (*E. pterophila*), a circle indicates the population on the Edwards Aquifer and the Blanco River system (*E. nana*), squares indicate populations on the Trinity Aquifer and the Guadalupe River system (*E. pterophila*), and stars indicate populations on the Edwards Aquifer and the Guadalupe River system (*E. neotenes*)

phased genotypes. The number of individuals analyzed for *ND4* and *rag1* variation is shown in Table 1 for each population. A maximum parsimony haplotype network was constructed for *ND4* and *rag1* datasets using TCS 1.2.1 (Clement et al. 2000), which employs the statistical algorithms of Templeton et al. (1992).

Data analysis

We used Fisher's exact test of population differentiation to determine if populations associated with different springs were genetically differentiated based on sequence data from *ND4* and *rag1*. This test was conducted using the software program Arlequin 2.000 (Schneider et al. 2000); 1,000 permutations were conducted to generate a distribution of test statistics under the null hypotheses of no genetic differentiation.

We used Hey and Nielsen's (2004) coalescent-based isolation with migration model as implemented in the software IM, which employs a Markov chain Monte Carlo (MCMC) algorithm to estimate the following parameters, each scaled to the neutral mutation rate: θ_1 and θ_2 (θ for populations 1 and 2, respectively), θ_A (θ of the ancestral population of 1 and 2), m (symmetrical migration rate), t (time of divergence), μ_1 and μ_2 (relative mutation rate of *ND4* and *rag1*, respectively) (Hey and Nielsen 2006). The isolation with migration model decomposes inter-population genetic similarity due to recent divergence from genetic similarity due to ongoing migration. This method differs from traditional methods for estimating inter-population migration rates, which assume that the populations have reached equilibrium between genetic drift and migration (Wright 1922; Slatkin 1987; Whitlock and McCauley 1999). The isolation with migration model assumes that sequence evolution occurs according to a neutral model and that no recombination occurs within loci. We found no evidence of deviations from neutral evolution for either *ND4* or *rag1* for any of the seven sampled populations using either Tajima's selective neutrality test or the Ewens–Watterson neutrality test ($P > 0.05$ for all populations for both loci), as implemented in Arlequin 2.000 (Schneider et al. 2000). As the *ND4* gene is from the mitochondrial genome it should not be subject to recombination. Furthermore, we found no evidence of recombination within the *rag1* gene based on the four-gamete test of Hudson and Kaplan (1985). As our data did not violate these assumptions, we proceeded with the IM analyses. Each locus was assigned an inheritance scalar, to adjust for its relative expected effective population size: 0.25 for *ND4* (mtDNA) and 1.0 for *rag1* (nuclear DNA). We used the infinite sites and HKY models of molecular evolution for the *ND4* and *rag1* genes,

respectively, based on the recommendations of Hey and Nielsen (2006). Each of the 21 pairwise analyses was performed multiple times using different priors and MCMC search conditions in order to identify run conditions that resulted in convergence. Analyses were considered to have converged upon the stationary distribution when independent runs using the same search conditions generated similar distributions for migration rate (m), relative mutation rates (μ_1, μ_2), and θ for the extant populations (θ_1, θ_2) and when the autocorrelation of parameter values decreased over the course of the run. In addition, we verified that parameter estimates did not show a directional trend over the post burn-in MCMC generations. The final priors and MCMC search conditions used for each pairwise analysis are given in Appendix A.

Similar to traditional methods that estimate pairwise migration rates based on genetic distances (e.g., pairwise F_{ST}; Wright 1922), the isolation with migration model assumes that a pair of populations exchanges migrants only with each other (Hey and Nielsen 2006). If a pair of populations exchanges migrants with additional populations, pairwise migration rate estimates may be inflated. This potential problem is unlikely to alter the findings of this study, as we fail to detect migration among the sampled *Eurycea* populations (see section "Results").

To determine if the observed patterns of genetic variation were more consistent with a model allowing migration than a model of complete isolation, we used the program IM to estimate the likelihood of the data for each pair of populations under a model allowing for migration and under a model with the migration rate equal to zero. We then calculated the likelihood ratio for these two models for each pair of populations. To test if the likelihood ratio was significantly greater than one, which would indicate that the model with migration explains the data significantly better than the model with the migration rate equal to zero, we compared the observed ratio to a composite distribution with equal parts taken from a chi-square distribution with one degree of freedom and a uniform distribution with a mean and variance of zero. This test procedure follows the suggestion of Nielsen and Wakeley (2001) when parametric bootstrapping is not feasible. The significance of likelihood ratio tests was assessed using R (R Development Core Team 2007).

To test for an association between geographic distances, hydrogeological features (i.e., rivers and aquifers) and genetic isolation among populations, we first calculated pairwise ϕ_{ST} values for both *ND4* and *rag1* for each pair of populations under the Jukes and Cantor model. The Jukes and Cantor model was selected because of the low level of sequence divergence detected among all sampled haplotypes. Pairwise ϕ_{ST} values were calculated in the program ARLEQUIN 2.000 (Schneider et al. 2000). The mean value of ϕ_{ST} for *ND4* and *rag1* for each pair of populations was obtained and used for all subsequent analyses. A Mantel test was then used to determine if genetic distance (ϕ_{ST}) was positively correlated with geographic distance. Geographic distances were estimated as straight-line distances. We then performed partial Mantel tests to determine if populations on different aquifers and/or river drainages were more genetically isolated than populations on the same aquifers and/or river drainages. Specifically, while controlling for geographic distance, we tested for a positive correlation between (1) genetic distance and aquifer distance ('aquifer distance' is a measure of dissimilarity with populations on different aquifers given a value of 1, and populations on same aquifer given a value of 0), and (2) genetic distance and river distance ('river distance' is a measure of dissimilarity with populations on different river systems given a value of 1, and populations on the same river system given a value of 0). Significance of correlations for Mantel and partial Mantel tests were assessed via 10,000 permutations using the R package CLUSTER (Maechler et al. 2005, unpublished data; R Development Core Team 2007).

Results

Fourteen and seven haplotypes were identified for *ND4* and *rag1*, respectively (Genbank accession numbers EF443108-EF443128; Table 1; Fig. 2). For *ND4*, a single haplotype (nA) was shared among the Jacob's Well (Trinity Aquifer/Blanco River), Ott's Spring (Trinity Aquifer/Guadalupe River), and Devil's Backbone (Trinity Aquifer/Guadalupe River) populations. Conversely for *rag1*, four haplotypes were shared among multiple populations and a single haplotype (rD) was found in populations on both aquifers and both rivers. The San Marcos Springs population (Edwards Aquifer/Blanco River) shared no haplotypes with any other populations for either locus.

We detected significant genetic differentiation using Fisher's exact test for all pairs of populations at the *ND4* locus and all pairs of populations except for Jacob's Well–Ott's Spring (both on Trinity Aquifer) at the *rag1* locus (Table 2). Interestingly, this was true for both intraspecific and interspecific population combinations.

While we were able to obtain reliable estimates of θ_1, θ_2, m, μ_1, and μ_2 for all pairs of populations, this was not true for θ_A and t (Fig. 3). However, this does not alter our confidence in our estimates of migration rate, as the simulations of Nielsen and Wakeley (2001) demonstrated that these parameters have little to no affect on estimates of migration rate. In addition, an inspection of likelihood

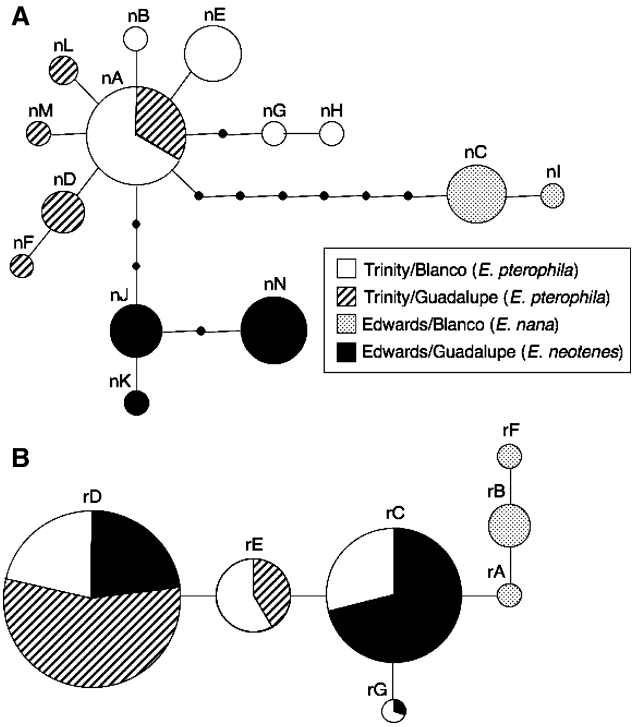

Fig. 2 Maximum parsimony network for *ND4* (mtDNA; **A**) and *rag1* (nuclear DNA; **B**). A circle represents each haplotype and the size of each circle is approximately proportional to the frequency of the haplotypes in the dataset. Haplotypes are marked as clear (Trinity Aquifer/Blanco River system, *E. pterophila*), striped (Trinity Aquifer/Guadalupe River system, *E. pterophila*), dotted (Edwards Aquifer/Blanco River system, *E. nana*), and solid (Edwards Aquifer/Guadalupe River system, *E. neotenes*) according to the proportion of individuals that belong to springs on the respective aquifer and river system

surfaces from our analyses indicates that we were able to obtain reliable estimates of our parameters of interest despite our inability to obtain estimates for θ_A and t (Fig. 3). Pairwise migration rate estimates under the isolation with migration model ranged from 0.002 to 5.250 m/μ (Table 3). Migration rates between San Marcos Springs and all other populations were particularly small (from 0.002 to 0.178 m/μ), while the migration rate between Devil's Backbone and Jacob's Well, both of which are on the Trinity Aquifer, was substantially larger (5.250 m/μ) (Table 3). For most of the 21 pairwise population combinations, the 95% confidence interval of the migration rate estimate was relatively small, but nonetheless the confidence interval overlapped with the point estimates for most of the other pairwise combinations (Table 3). For all 21 pairwise population combinations, we failed to reject the null model of no migration when compared to a model allowing migration using likelihood ratio tests.

Mean pairwise genetic distances (ϕ_{ST}) based on both *ND4* and *rag1* ranged from 0.249 to 0.924. In most cases ϕ_{ST} was greater than 0.5, indicating that the majority of genetic variation was partitioned among populations. In concordance with migration rate estimates under the isolation with migration model, pairwise genetic distances between San Marcos Springs and all other populations were relatively large (between 0.845 and 0.924), while the genetic distance between Devil's Backbone and Jacob's Well was much smaller (0.249) (Table 3). In general, pairs of populations with higher estimates of genetic distance had lower migration rate estimates (Table 3).

There was a marginally significant positive correlation between genetic and geographic distance ($r = 0.385$, $P = 0.057$; Fig. 4a). However, while controlling for geographic distance, we did not detect a positive correlation between genetic distance and aquifer distance ($r = 0.214$, $P = 0.124$; Fig. 4b), or genetic distance and river distance ($r = -0.372$, $P = 1.000$; Fig. 4c). In fact, the correlation

Table 2 *P*-values from Fisher's exact test of population differentiations

	Jacob's Well (T/B)	Ott's Spring (T/G)	Devil's Backbone (T/G)	Fern Bank (T/B)	Comal Springs (E/G)	Hueco Springs (E/G)	San Marcos Springs (E/B)
Jacob's Well (T/B)	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Ott's Spring (T/G)	0.999	–	<0.001	<0.001	<0.001	<0.001	<0.001
Devil's Backbone (T/G)	0.005	0.021	–	<0.001	<0.001	<0.001	<0.001
Fern Bank (T/B)	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001
Comal Springs (E/G)	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001
Hueco Springs (E/G)	0.044	0.042	<0.001	<0.001	<0.001	–	<0.001
San Marcos Springs (E/B)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–

Values below the diagonal are for *rag1* and values above the diagonal are for *ND4*. Letters in parentheses following population names indicate the aquifer and river where the population is found; 'T' represents the Trinity Aquifer, 'E' represents the Edwards Aquifer, 'B' represents the Blanco River, and 'G' represents the Guadalupe River

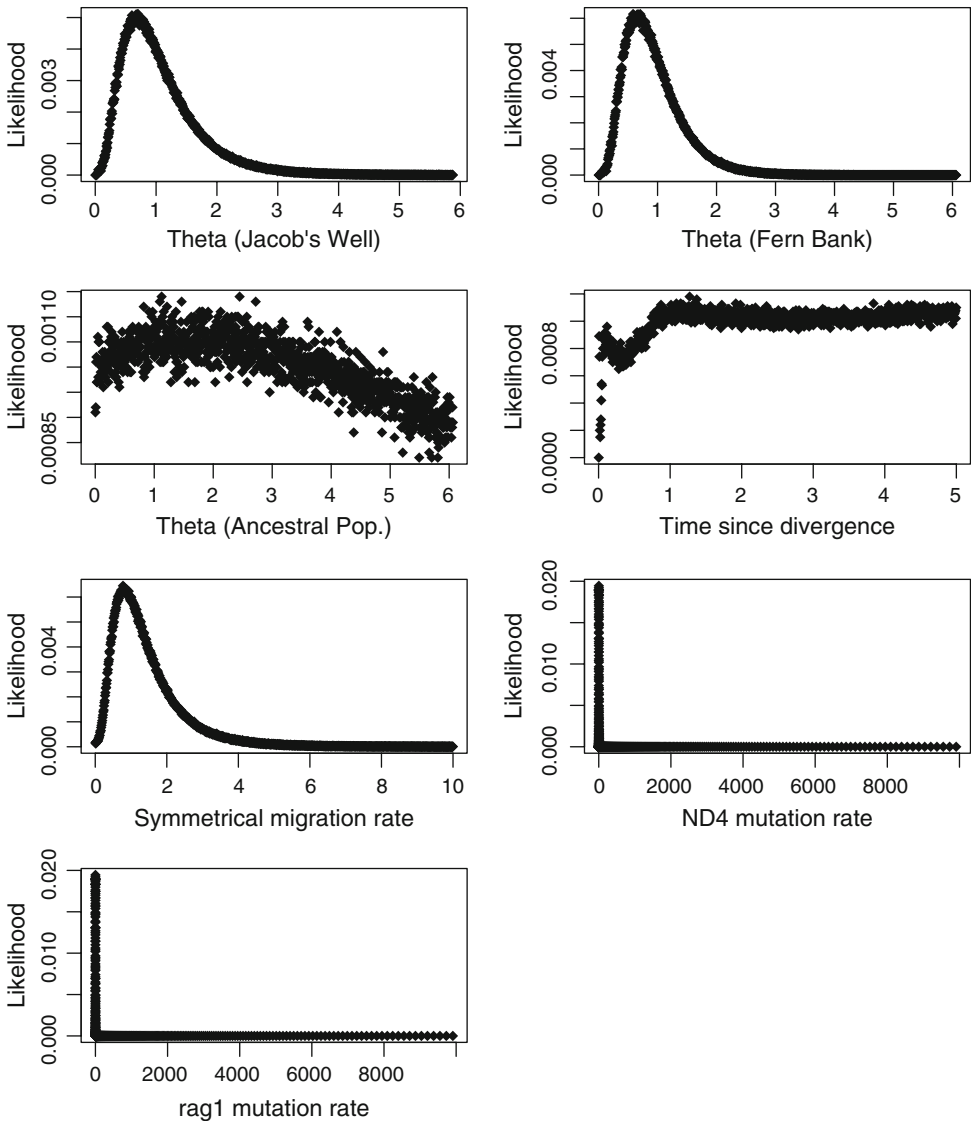

Fig. 3 Likelihood surfaces for θ_1, θ_2, θ_A, m, t, μ_1, and μ_2 from the IM analysis under a model allowing for migration with the Jacob's Well and Fern Bank populations. The results shown here involving these populations are indicative of our general ability to reliably estimate each model parameter for each pair of populations

coefficient for the partial Mantel test involving river distance was negative.

Discussion

Population isolation

Our results indicate that the sampled Edwards Plateau *Eurycea* (*Blepsimolge* clade) populations are genetically isolated and experience little or no inter-population migration. All of the sampled *Eurycea* populations contain at least one private allele at the mitochondrial *ND4* locus, and the San Marcos Springs population possesses private alleles at the nuclear *rag1* locus (Table 1). Consistent with these findings, nearly all pairs of populations are genetically differentiated based on Fisher's exact test of population differentiation (Table 2). Additionally, migration rate estimates are generally low for all pairs of populations (Table 3). Finally, and in agreement with the above, we are unable to reject a model of complete isolation for any pair of populations. The estimates of migration rate and genetic distance from this study are similar to those observed in other endemic and/or geographically isolated taxa (Chenoweth et al. 1998; Townsend et al. 2007). For Edwards Plateau *Eurycea*, genetic isolation is not limited to population comparisons involving populations assigned to different species, but includes pairs of populations assigned to the same species (e.g., the Jacob's Well and Fern Bank populations of *E. pterophila*). Thus, based on the current taxonomy, substantial interspecific and inter-population genetic differentiation exists in Edwards Plateau *Eurycea*.

Table 3 Pairwise non-equilibrium migration rates (m/μ) and their 95% confidence intervals from IM (lower diagonal) and pairwise ϕ_{ST} estimates (mean of estimates from *ND4* and *rag1*) (upper diagonal)

	Jacob's Well (T/B)	Ott's Spring (T/G)	Devil's Backbone (T/G)	Fern Bank (T/B)	Comal Springs (E/G)	Hueco Springs (E/G)	San Marcos Springs (E/B)
Jacob's Well (T/B)	–	0.368	0.249	0.590	0.896	0.542	0.908
Ott's Spring (T/G)	2.490 (0.330–15.390)	–	0.470	0.662	0.909	0.536	0.909
Devil's Backbone (T/G)	5.250 (0.250–87.250)	2.415 (0.315–18.305)	–	0.658	0.922	0.618	0.924
Fern Bank (T/B)	0.775 (0.175–2.755)	0.583 (0.068–2.268)	0.503 (0.063–1.868)	–	0.518	0.631	0.845
Comal Springs (E/G)	0.308 (0.018–1.138)	0.005 (0.005–0.555)	0.005 (0.005–0.545)	0.563 (0.038–1.973)	–	0.774	0.887
Hueco Springs (E/G)	1.055 (0.215–4.475)	0.353 (0.003–1.663)	0.373 (0.003–1.878)	0.815 (0.155–3.045)	0.748 (0.088–2.643)	–	0.882
San Marcos Springs (E/B)	0.003 (0.003–0.403)	0.002 (0.002–0.311)	0.002 (0.002–0.305)	0.003 (0.003–0.283)	0.178 (0.003–0.703)	0.002 (0.002–0.362)	–

Letters in parentheses following population names indicate the aquifer and river where the population is found; 'T' represents the Trinity Aquifer, 'E' represents the Edwards Aquifer, 'B' represents the Blanco River, and 'G' represents the Guadalupe River

Pairwise migration rate estimates involving the San Marcos Springs population (*E. nana*), which is listed federally as threatened in the USA (USFWS 1980), are particularly low (Table 3). This is consistent with the fact this population is fixed for unique alleles at both *ND4* and *rag1* (Table 1). These data support earlier studies that suggest that the San Marcos Springs *E. nana* population is relatively highly divergent from other Edwards Plateau populations (Chippindale 1998; Chippindale et al. 2000; Hillis et al. 2001).

The positive correlation between geographic and genetic distance we detected is consistent with a model of isolation by distance. Such patterns of isolation by distance are often detected in species with limited dispersal ability and/or fragmented habitats (Chenoweth et al. 1998; Segelbacher et al. 2003; Cook et al. 2008). We have little to no evidence that gene flow occurs among the sampled *Eurycea* populations and the populations' habitats are intrinsically isolated, thus, both of these factors apply to Edwards Plateau *Eurycea*. Our failure to reject a null model of no migration for any pair of populations in conjunction with our support for an isolation by distance model, raises the possibility that populations separated by less geographic distance became physically isolated more recently than those separated by greater geographic distance.

We failed to detect a positive association between genetic distance and aquifer or river distance. There are several explanations for these findings. First, it is possible that an insufficient number of populations were included in this study to provide adequate power to detect the potential influence of hydrogeological features on genetic isolation. Alternatively, inter-population migration may be sufficiently rare and stochastic to preclude a strong relationship between genetic and hydrogeological isolation. This latter scenario is consistent with the low levels of inter-population migration and high degree of genetic isolation detected in this study. In addition, changes in flow patterns of hydrogeological features through time may obscure any potential relationship between these hydrogeological features and genetic distances between pairs of populations. A final possibility is that the majority of migration events occur during periodic floods along ephemeral aquatic connections; such floods are known to occur in the Edwards Plateau region semi-regularly (Baker 1975). These possibilities are not necessarily mutually exclusive and may all have contributed to our inability to detect a positive association between genetic distance and aquifer or river distance.

Taxonomic and conservation implications

In general, our data are consistent with but do not fully corroborate the current taxonomy. Populations belonging to the same species normally have higher migration rate estimates and lower genetic distances than populations belonging to different species. However, there are exceptions to this generality. For example, the migration rate estimate between the Fern Bank and Jacob's Well populations (both *E. pterophila*) was lower than the migration estimate for Hueco Springs (*E. neotenes*) and Jacob's Well (Table 3). Furthermore, a discrete boundary

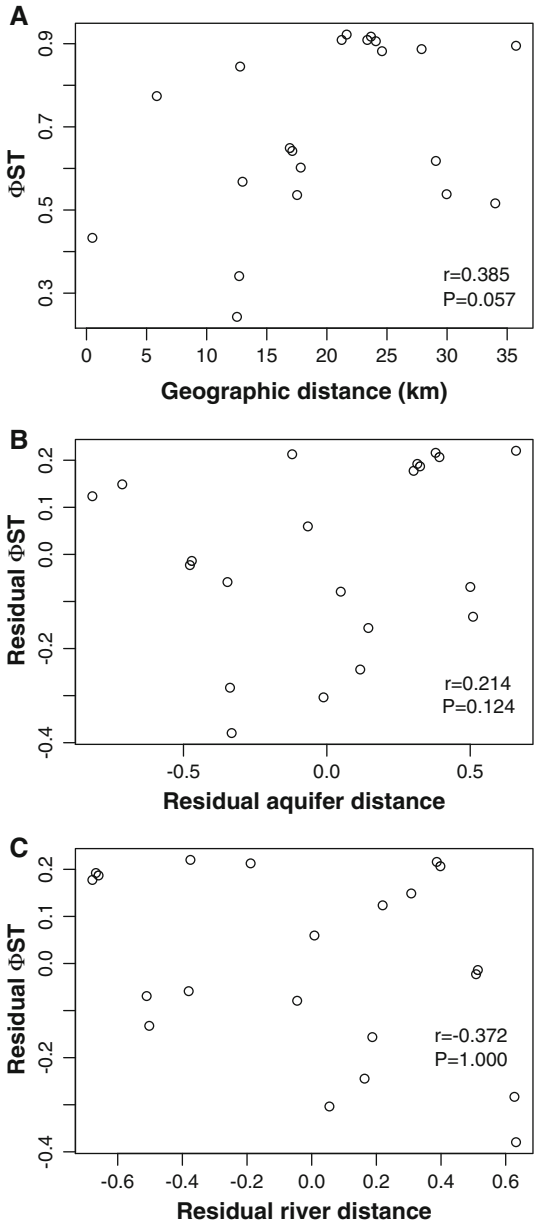

Fig. 4 Scatter plot depicting the correlation between genetic distance (ϕ_{ST}) and geographic distance (**A**), and scatter plots of residuals from the partial Mantel tests depicting the correlation between genetic distance and aquifer (**B**) or river distance (**C**) while controlling for geographic distance

between intra-specific migration rate estimates and/or genetic distance measures and inter-specific migration rate estimates and/or genetic distances was not apparent based on our results as shown in Tables 1 and 3. Future taxonomic revisions of Edwards Plateau *Eurycea* should consider the haplotype sharing among Jacob's Well (*E. pterophila*), Ott's Spring, and Devil's Backbone, as the literature suggests *E. pterophila* are only found on the Blanco River drainage (Hillis et al. 2001), while Ott's Spring and Devil's Backbone populations occur on the Guadalupe River drainage.

The low migration rate estimates observed among all pairs of sampled populations indicate that evolutionary and ecological processes occurring in any one population likely have little impact on the evolutionary and ecological processes occurring in other populations. This finding has two implications. First, our evidence of little or no gene flow among *Eurycea* populations indicates that each of these populations is likely on an independent evolutionary trajectory, determined primarily by local abiotic and biotic conditions as well as stochastic factors (i.e., novel mutations and genetic drift) (Moritz 1999). Therefore, each of the seven populations sampled in this study qualifies for consideration as a distinct management unit. Second, because the sampled *Eurycea* populations experience little inter-population migration, we cannot assume populations that become extirpated have a high probability of being re-colonized by migrants from other populations. Edwards Plateau *Eurycea* do not appear to represent a meta-population with frequent episodes of local extinction followed by re-colonization.

As the Edwards Plateau contains many additional springs similar to the seven sampled in this study, these results need to be viewed with a degree of caution. In some cases springs exist between the sites we sampled. These unsampled intervening springs may exchange migrants with some of the sampled sites. Thus, future studies incorporating these additional springs could strengthen and/or modify the conclusions that have emerged from the study of *Eurycea* populations to date. The core findings from the current study, that Edwards Plateau *Eurycea* are geographically and genetically isolated, provide the starting point for future investigations.

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Appendix

Appendix A Optimized IM input parameters for pairwise population comparisons

Pairwise population	IM input parameters
Jacobs Well–Ott's Spring	-q1 20 -m1 60 -m2 60 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Jacob's Well–Devil's Backbone	-q1 20 -m1 500 -m2 500 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Jacob's Well–Fern Bank	-q1 5 -m1 10 -m2 10 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Jacob's Well–Comal Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Jacob's Well–Hueco Springs	-q1 5 -m1 10 -m2 10 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Jacob's Well–San Marcos Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Ott's Spring–Devil's Backbone	-q1 70 -m1 70 -m2 70 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Ott's Spring–Fern Bank	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Ott's Spring–Comal Springs	-q1 10 -m1 10 -m2 10 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Ott's Spring–Hueco Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Ott's Spring–San Marcos Springs	-q1 3 -m1 3 -m2 3 -t5 -j7 -p45 -b1000000 -L10000000 -fg -g10.9 -g20.75 -n20 -k20
Devil's Backbone–Fern Bank	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Devil's Backbone–Comal Springs	-q1 10 -m1 10 -m2 10 -t5 -j7 -p45 -b1500000 -L15000000 -fg -g10.9 -g20.75 -n30 -k30
Devil's Backbone–Hueco Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Devil's Backbone–San Marcos Springs	-q1 3 -m1 3 -m2 3 -t5 -j7 -p45 -b100000 -L10000000 -fg -g10.9 -g20.75 -n20 -k20
Fern Bank–Comal Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.7 -n20 -k20
Fern Bank–Hueco Springs	-q1 5 -m1 10 -m2 10 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Fern Bank–San Marcos Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Comal Springs–Hueco Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Comal Springs–San Marcos Springs	-q1 5 -m1 5 -m2 5 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20
Hueco Springs–San Marcos Springs	-q1 3 -m1 3 -m2 3 -t5 -j7 -p45 -b1200000 -L12000000 -fg -g10.9 -g20.75 -n20 -k20

Refer to IM manual for description of parameters

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