



Phylogenetic structure of *Holbrookia lacerata* (Cope 1880) (Squamata: Phrynosomatidae): one species or two?

TOBY J. HIBBITTS^{1,2}, WADE A. RYBERG², JOHANNA A. HARVEY¹, GARY VOELKER¹,
A. MICHELLE LAWING³, CONNOR S. ADAMS², DALTON B. NEUHARTH²,
DREW E. DITTMER⁴, C. MICHAEL DURAN⁵, BRAD D. WOLAVER⁶, JON PAUL PIERRE⁶,
BENJAMIN J. LABAY^{7,†} & TRAVIS J. LADUC⁷

¹Biodiversity Research and Teaching Collection, Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843-2258

²Natural Resources Institute, Texas A&M University, College Station, TX 77843

³Department of Ecosystem Science and Management, Texas A&M University, College Station TX 77843-2120

⁴Texas Tech University, Department of Natural Resources Management, 2500 Broadway, Lubbock, Texas 79409

⁵The Nature Conservancy, San Antonio, TX 78215,

⁶Bureau of Economic Geology, Jackson School of Geosciences, The University of Texas at Austin, Austin, Texas 78758, USA

⁷Biodiversity Collections, Department of Integrative Biology, The University of Texas at Austin, Austin, TX 78758-4445

† Benjamin J. Labay currently at Siglo Group, Austin, TX 78702

Abstract

Species delimitation attempts to match species-level taxonomy with actual evolutionary lineages. Such taxonomic conclusions are typically, but not always, based on patterns of congruence across multiple data sources and methods of analyses. Here, we use this pluralistic approach to species delimitation to help resolve uncertainty in species boundaries of phrynosomatid sand lizards of the genus *Holbrookia*. Specifically, the Spot-tailed Earless Lizard (*H. lacerata*) was historically divided into a northern (*H. l. lacerata*) and southern (*H. l. subcaudalis*) subspecies based on differences in morphology and allopatry, but no research has been conducted evaluating genetic differences between these taxa. In this study, patterns in sequence data derived from two genes, one nuclear and one mitochondrial, for 66 individuals sampled across 18 counties in Texas revealed three strongly supported, reciprocally monophyletic lineages, each comprised of individuals from a single geographic region. Distinct genetic variation evident across two of these regions corresponds with differences in morphology, differences in environmental niche, and lines up with the presumed geographic barrier, the Balcones Escarpment, which is the historical subspecies boundary. The combined evidence from genetics, morphology and environmental niche is sufficient to consider these subspecies as distinct species with the lizards north of the Balcones Escarpment retaining the name *Holbrookia lacerata*, and those south of the Balcones Escarpment being designated as *Holbrookia subcaudalis*.

Key words: *Holbrookia lacerata*, *Holbrookia subcaudalis*, Texas, Balcones Escarpment, ND2, RAG-1, morphology, environmental niche

Introduction

Delimiting species is a fundamental function of taxonomy. While many methods have been used for species delimitation (reviewed by Carstens *et al.* 2013), it is critically important to investigate multiple sources of data for each potential species (Knowles & Carstens 2007; Schlick-Steiner *et al.* 2010). Congruence of all data sources is not necessary when delimiting species. For example, morphology is known to be conservative (and thus less informative) in many taxa with deep genetic divergences. However, congruence between genetic and non-genetic data sources (e.g., life history, distribution, morphology, ecology, and behavior) provides a compelling argument for concluding that populations or metapopulations are their own evolutionary lineage (General Lineage Concept—de Queiroz 1998).

Using multiple methods and criteria for species delimitation would be particularly helpful in resolving uncer-

tainty in species boundaries of phrynosomatid lizards and especially those belonging to the sand lizards. This group is comprised of 12 currently recognized species belonging to four different genera: *Uma*, *Callisaurus*, *Cophosaurus*, and *Holbrookia* (Schulte & de Queiroz 2008; Wilgenbusch & de Queiroz 2000). Although the monophyly of this group, its relationships to other phrynosomatid lizards, and the monophyly of the four genera listed above are well-supported (de Queiroz 1992; Schulte & de Queiroz 2008; Wiens *et al.* 2010; Wilgenbusch & de Queiroz, 2000), the taxonomic designation of lineages within some of those genera are unresolved. The genus *Holbrookia*, for example, contains the following four “earless” species (i.e., with a concealed tympanic membrane): *H. elegans* Bocourt (2 subspecies), *H. lacerata* Cope (2 subspecies), *H. maculata* Girard (5 subspecies), and *H. propinqua* Baird and Girard. Phylogenetic relationships, geography, and patterns of morphological variation suggest that some of the subspecies may warrant species rank (Wilgenbusch & de Queiroz, 2000).

For the Spot-tailed Earless Lizard (*Holbrookia lacerata*) in particular, two subspecies have been distinguished based on allopatry and morphology (Axtell 1956, 1968). The distributions of these subspecies are clearly separated by the southern edge of the Edwards Plateau, or Balcones Escarpment (Fig. 1; Axtell 1956, 1968). The southern subspecies (*H. l. subcaudalis*) differs from the northern subspecies (*H. l. lacerata*) in average adult size (snout-vent length 62 mm vs. 54 mm, respectively) and average femoral-pore count (15.7 to 12.8, respectively; Axtell 1956, 1968). The southern subspecies also has unfused pairs of blotches on each side of the dorsal vertebral line and rounded blotches on the hind legs, rather than fused dorsal blotches and dark bands on the hind legs found in the northern subspecies (Fig. 1). Despite these clear geographic and morphological differences, only recently have researchers become interested in determining whether genetic differences between *H. lacerata* subspecies exist (Roelke *et al.* 2018).

In this study, we evaluate the subspecies designations of *Holbrookia lacerata* using sequence data derived from two genes, one nuclear and one mitochondrial, morphological data, and ecological niche modeling. Our goal was to provide information about genetic diversity, morphological differentiation, and niche differences within *H. lacerata*. This will determine congruence among data types and provide empirical support for delimitation of subspecies within *H. lacerata*. Results of this research have implications for future conservation and management of *H. lacerata* populations, as this species is currently being considered for listing by the U.S. Fish and Wildlife Service (FWS) under the Endangered Species Act (USFWS 2011).

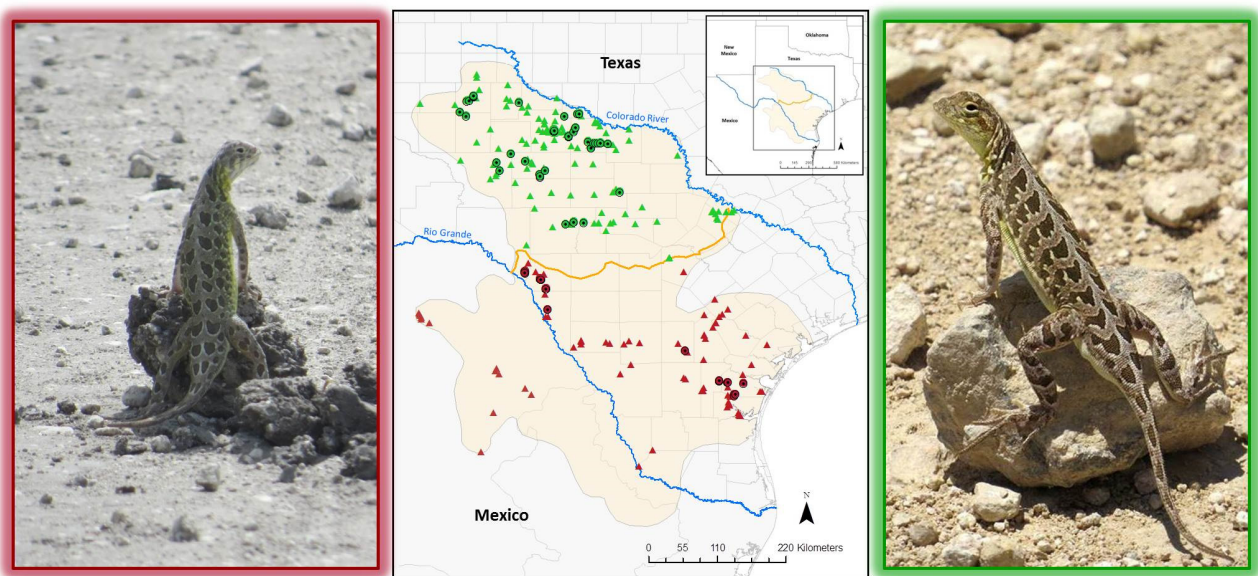


FIGURE 1. The shaded portion of the map depicts the historical distribution of *Holbrookia lacerata* (IUCN). The green and red triangles are localities for the historically divided northern (*H. l. lacerata*) and southern (*H. l. subcaudalis*) subspecies, respectively. The samples collected for this study are depicted as red or green circles. The orange line represents the Balcones Escarpment. The photograph outlined in red is a male *Holbrookia subcaudalis* from Jim Wells County, Texas and the photograph outlined in green is a male *H. lacerata* from Schleicher County, Texas. Photographs by Toby J. Hibbitts.

Methods

Phylogenetics. We collected tissue samples during 274 statewide surveys in Texas between April and September 2015. We did most of these surveys by driving roads, which has proven to be an effective method for collecting *Holbrookia lacerata* (TJH, WAR pers obs). We took liver samples from up to two specimens at each survey site. We also collected tail tips from additional captured lizards and collected tissues from lizards found dead on the road. We contacted natural history collections and museums to supplement tissues collected from road searches. In total, we amassed 73 tissues from three *Holbrookia* species, which included 66 *H. lacerata* samples from 20 Texas counties.

We extracted whole genomic DNA from tissues, tail tips, and blood as available using the E.Z.N.A Tissue Extraction Kit and standard protocols (Omega Bio-Tek, Norcross, GA) for *Holbrookia lacerata* individuals as well as from two outgroups (*H. maculata* (n = 4) and *H. propinqua* (n = 3)). We then sequenced a mitochondrial and a nuclear gene. We selected the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) as it was informative in reconstructing diversification patterns within the sand lizard clade (*Uma*, *Callisaurus*, *Cophosaurus*, and *Holbrookia*) of Phrynosomatidae (Blaine 2008). We amplified 1,086 bp of ND2 using primers H4437 and Ala.r3 (Blaine 2008; Macey *et al.* 1997), for all samples. The nuclear gene amplified was the Recombination Activating Gene 1 (RAG-1), of which we amplified for 33 *Holbrookia* individuals (including one each of *H. maculata* and *H. propinqua* as outgroups) and 1,054bp using JRAG1f2 and JRAG1r3 (Leaché & McGuire 2006). We selected RAG-1 as it has proven phylogenetically informative in resolving relationships within other phrynosomatid genera (Wiens *et al.* 2010).

We performed polymerase chain reactions (PCR) with GoTaq Flexi DNA polymerase (Promega). Automated sequencing was performed using BigDye (Applied Biosystems) and products were sequenced on an ABI PRISM 3730xl (Beckman Coulter, Danvers, MA). We verified and aligned sequences by eye using Geneious 6.1.8 (<http://www.geneious.com>, Kearse *et al.*, 2012). We used PHASE 2.1 (Stephens *et al.* 2001) for haplotype reconstruction of diploid gametic alleles for the RAG-1 gene as implemented in the DnaSP 5.10.1 package (Librado & Rozas 2009). We used a 1000 burn-in, 10 thinning intervals, and 1000 main iterations resulting in 66 sequences for 33 taxa.

Phylogenetic analyses were first conducted on single gene trees. The most appropriate models of evolution were determined using Partition Finder 2.1.1 (Lanfear *et al.* 2012, Lanfear *et al.* 2017) for each single gene assessed across all positions. For the ND2 gene, the most appropriate model selected using the Akaike information criterion (AIC) was GTR + G, whereas the Bayesian information criterion (BIC) criterion selected the HKY + G. For the RAG-1 gene, the model selected as most appropriate under AIC and BIC criterion was HKY + I for all positions. Bayesian inference (BI) was conducted using MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003) and in two Monte Carlo Markov Chain (MCMC) analyses (Geyer 1991) which were run for 10 million generations and sampled every 1000. For ND2, we ran both AIC and BIC selected models (GTR + G and HKY + G) and for RAG-1, we ran the AIC and BIC selected model (HKY + I) as well as a reversible jump (RJ) analysis. For all analyses, convergence and appropriate burn-in was assessed using Tracer (Rambaut 2007) and potential scale reduction factors were verified to have reached convergence values (PSRF=1.0). Maximum likelihood (ML) analysis was conducted using RAxML 7.2.7 (Stamatakis 2006) on the CIPRES Science Gateway Portal (Miller *et al.* 2010). Analyses were run under the GTRGamma model and all parameters were estimated. Rapid bootstrap support values were estimated with 10,000 replicates.

Concatenated gene analysis was run in MrBayes 3.2.6 (BI) and RAxML 7.2.7 (ML) respectively. For BI concatenated gene tree analysis, the most appropriate models selected using AIC was 3 partitions, ND2 position one under GTR + I, ND2 position three under HKY + I, and RAG1 all positions under HKY + I + G. The most appropriate model selected using the BIC criterion was HKY + I + G for both genes across all positions. ML concatenated analysis was run under the GTRGamma model with all parameters estimated and rapid bootstrap values estimated with 10,000 replicates.

We constructed median-joining haplotype networks using Network 5.0 (Fluxus-engineering.com) to discern patterns across different haplotype genotypes. Uncorrected pair-wise sequence divergences among mtDNA haplotype groups was determined using MEGA7.0.14 (Kumar *et al.* 2016) for the ND2 gene.

Morphology. We amassed 157 *H. lacerata* specimens (*H. l. lacerata* = 112; *H. l. subcaudalis* = 45) from surveys and natural history museums. On each lizard we measured snout-vent length (SVL), tail length, head width, head depth, head length, upper arm length (humerus), lower arm length (from elbow to longest finger), upper leg length (femur), lower leg length (tibia), foot length (foot to longest toe), fourth toe length, and interlimb length to

the nearest millimeter using digital calipers. We also counted the number of lateral spots, body blotches, connected blotches (between pelvic and pectoral girdles), femoral pores (left and right separately), and blotches on right leg (Fig 2).

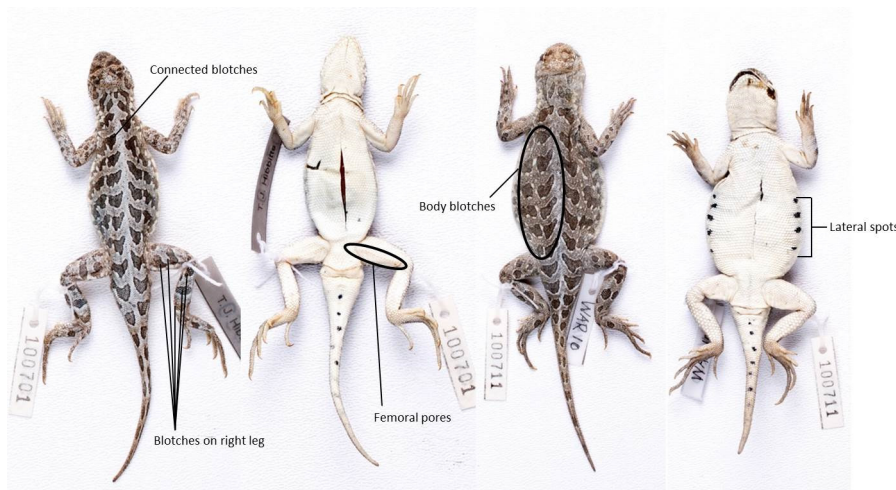


FIGURE 2. Morphological characters used to distinguish northern and southern subspecies from one another. The two images on the left are from a single northern (*H. l. lacerata*) male (dorsal and ventral view). The two images on the right are from a single southern (*H. l. subcaudalis*) female (dorsal and ventral view).

We removed the effects of body size by taking the residuals from linear regressions against SVL for all length measurements. We used these residuals in all subsequent analyses. We investigated morphological variables for multicollinearity. We found significant pairwise Pearson correlation between each of the leg variables (femur, tibia, foot, and fourth toe) and arm variables (humerus and lower arm), therefore we used only femur and humerus length in the analysis. We performed t-tests on the variables commonly associated with sexual dimorphism in phrynosomatid lizards (SVL, head width, head depth, head length, and interlimb length). We found that head width ($t = 2.19$, $p = 0.03$), head depth ($t = 2.28$, $p = 0.02$), and interlimb length ($t = -7.708$, $p < 0.001$) were all significantly different between sexes; therefore, we did not use these sexually dimorphic characters in later analyses.

We used principle component analysis (PCA) to investigate the importance of morphometric variables and to visualize the morphospace occupied by the two *H. lacerata* subspecies. We used t-tests, with Bonferroni correction, to test for significance of each variable between subspecies. All statistical analyses were done in the program PAST 3.07 (Hammer and Harper, 2006).

Ecological Niche Correlates. To infer whether there is ecological divergence between the two subspecies of *H. lacerata*, we tested for niche equivalency and background similarity (Warren *et al.* 2008). Niche equivalency tests whether two modeled ecological niches are more different than expected if drawn from the same population. This test uses Schoener's (1968) statistic for niche overlap (D) and a similarity statistic (I) based on Hellinger distances (van der Vaart 1998). Both statistics range from 0 to 1, where 0 indicates no overlap and 1 indicates complete overlap. A randomization procedure partitions the occurrences from known groups into randomly assigned groups and then uses the groups to estimate ecological niche models. D and I are calculated for each permutation of this procedure and are compared to the original D and I for the known groups. Background similarity tests whether the difference between two modeled ecological niches are more similar than expected, given the difference between their backgrounds (i.e., available or accessible environments). We used 1000 permutations in both tests of niche equivalency and background similarity. Tests were performed in the R Statistical Programming Language with the packages *ecospat* (Di Cola *et al.* 2017), *maxent* (Phillips *et al.* 2017), *SDMTools* (VanDerWal *et al.* 2014), and *dismo* (Hijmans *et al.* 2017). We compiled occurrence data and climate variables to build ecological niche models for tests of niche equivalency and background similarity.

Occurrence data ($n = 565$) were compiled from all known locality records of *H. lacerata* (Fig. 1). Occurrences were separated into *H. l. lacerata* ($n = 322$) and *H. l. subcaudalis* ($n = 243$) based on morphology, genetics, and/or geographic location of sample. To account for spatial and collector bias in the occurrence data, we used an environmental filtering method that down samples occurrences based on gridding predictor variable space (Varela *et al.* 2014). The environmental filtering procedure randomly samples one occurrence point from each equidistant grid

cell in predictor variable space. Each variable grid was based on equidistant spacing of grid cells. After applying the environmental filter, the occurrence dataset was reduced to 122 occurrences for *H. l. lacerata* and 64 occurrences for the *H. l. subcaudalis*.

Predictor variables included in the analysis were from a suite of bioclimatic variables in the Worldclim 2 database sampled at a 2.5 minute resolution (Fick and Hijmans 2017). Bioclimatic variables are meant to be biologically meaningful and represent means and extremes of temperature and precipitation at various temporal scales (monthly, quarterly, and yearly) (Nix 1986). We removed strongly correlated variables ($r > 0.65$ or $r < -0.65$) from the suite of nineteen bioclimatic variables to simplify interpretation of variable contribution and permutation importance results from ecological niche modeling, discussed below. Five predictor variables met the criteria: mean annual temperature (C), isothermality (%), maximum temperature of the warmest month (C), annual precipitation (mm), and precipitation seasonality (%). Isothermality is a ratio that compares the average day to night temperature differentials with annual temperature differentials; if the ratio is 1 (or 100%), then the difference in temperature during an average day or month is no different than the difference in temperature between seasons. Precipitation seasonality is the coefficient of variation of monthly precipitation. For detailed descriptions of the derivation of these five variables see Nix (1986).

We used Maxent version 3.4.1 to build ecological niche models of *H. l. lacerata* and *H. l. subcaudalis* (Phillips *et al.* 2017). We randomly sampled 20% of occurrence records after filtering for a testing dataset and used the remaining 80% of data for model training. Order of variable importance and amount of variable contribution was quantified for each model. We used area under the receiving operating characteristic curve (AUC) to evaluate training and testing datasets (Fielding and Bell 1997). In general, an AUC of 0.75 is considered informative (Elith *et al.* 2006); although, the higher the AUC, the better fit the model. Although ecological niche models will show whether there is projected overlap in the distributions of *H. l. lacerata* and *H. l. subcaudalis*, the results do not distinguish between background differences and their influence on the models. To consider this, we used tests for niche equivalency and background similarity.

Results

Phylogenetics. We recovered 2156 bp for both loci (1002 bp for ND2 and 1054 bp for RAG1). No gaps or indels were found within amplicons. For ND2 mtDNA, BI analysis was run under HKY + I and GTR + I models of evolution and ML analysis was run under GTRGamma. These resulted in highly congruent phylogenies across BI and ML analyses. The topologies recover two monophyletic, fully supported clades (posterior probability = 1.0, bootstrap support = 100; Fig. 3). These clades represent *H. lacerata* individuals from north of the Balcones Escarpment and *H. lacerata* individuals from south of the Balcones Escarpment. Within the southern clade, two subclades were recovered (posterior probability ≥ 0.95 , bootstrap support ≤ 95 ; Fig. 3) corresponding to southwest and southeast Texas, respectively.

Haplotype network analysis for ND2 recovered 38 unique haplotypes from 66 samples included in the analysis. These haplotypes cluster into three distinct haplogroups, which correspond to distinct geographic regions (north, southwest, southeast; Fig. 3). We observed no regions with shared haplotypes (Fig. 3). The northern haplotype group was over 90 mutational steps from the most genetically similar southwestern haplotype, and over 70 from the most genetically similar southeastern haplotype. The southern haplotype groups differed by over 30 mutational steps.

Uncorrected pairwise (p) genetic distance, for the ND2 gene, between three clades/haplogroups was greatest between the north clade, and the southwest and southeast clades (8.70% and 7.60%, respectively). P -distance between the southwest and southeast clades was 3.20%.

The BI and ML analysis, run for selected models for *Holbrookia lacerata* RAG-1 nuclear DNA gene all resulted in highly supported polytomy (posterior probability ≥ 0.95 , bootstrap support ≥ 95) uniting all *H. lacerata* individuals relative to outgroup taxa (Fig. 4). Topologies across all analysis were all concordant. The network analysis contained 66 phased sequences (in group taxa only) and resulted in 36 distinct haplotypes. Unlike our results for ND2, the RAG-1 gene does not show discrete clustering wherein all individuals were reciprocally monophyletic by geography (north, southwest, or southeast), although some internal clades do show some geographic structuring (Fig. 4). However, within these internal subclades, there is only one instance of a haplotype being shared between geographic regions (southwest + southeast; Fig. 4).

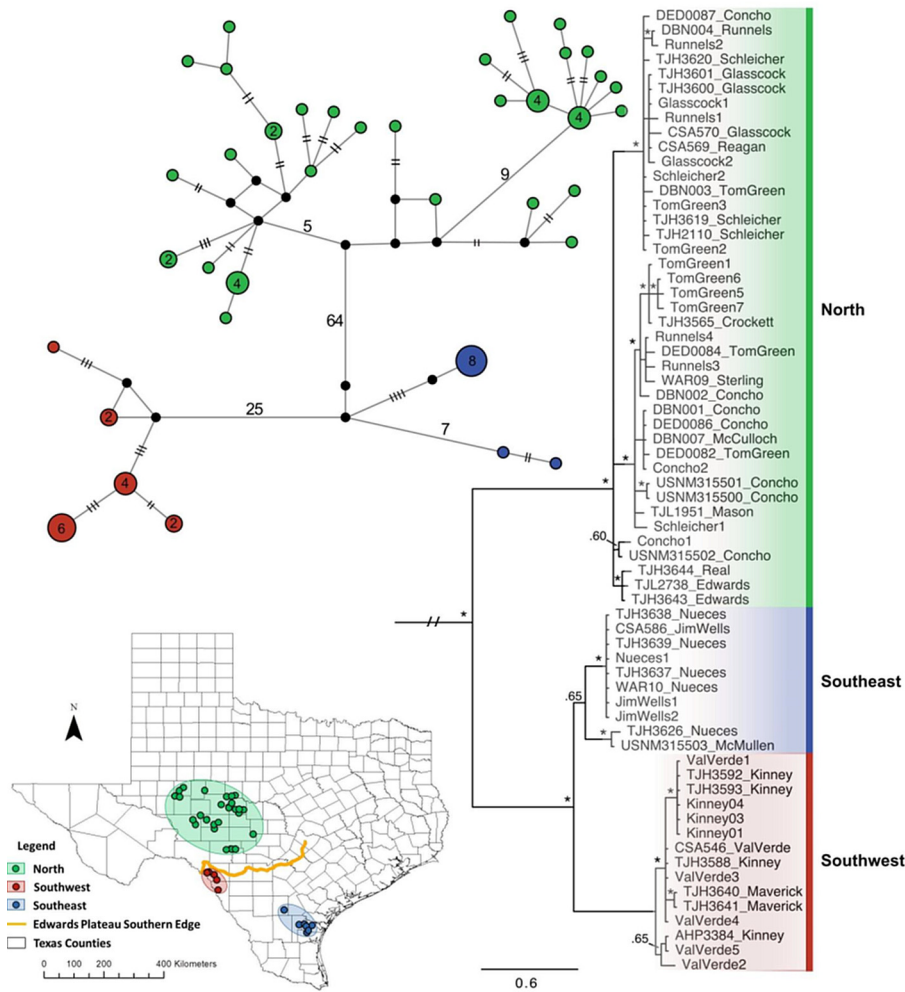


FIGURE 3. The map depicts the locations of tissue samples used in the analysis. In all cases the northern species (*Holbrookia lacerata*) is green. The southwestern population of the southern species (*Holbrookia subcaudalis*) is red and the southeastern population of the southern species is blue. Median-joining network (left) of the observed 38 haplotypes for ND2 mtDNA gene sequences of 66 spot-tailed earless lizard individuals. Circle sizes are proportional to frequencies of haplotypes. Black circles indicate missing intermediates (unsampled). Cross-hatches represent mutational steps with all greater than four denoted by the number of steps. Bayesian analysis of the mtDNA ND2 gene (right). Numbers at the nodes are posterior probability values. Asterisks indicate values = 1.

The Bayesian analysis for the concatenated gene trees under all models yielded identical topologies and similar support values at all nodes (Supplemental). The topology recovered was consistent with our ND2 analysis, resulting in two well-supported clades (posterior probability = 1.0 and bootstrap = 100), one comprised of *Holbrookia lacerata* individuals from north of the Balcones Escarpment and one comprised of individuals to the south. The southern clade is subdivided into the southwest and southeast individuals of *H. lacerata* (posterior probability ≥ 0.95 and bootstrap = ≤ 95 ; Supplemental).

Morphology. We used 112 adult *H. l. lacerata* (northern) and 45 adult *H. l. subcaudalis* (southern) for our morphometric analysis. We found that the northern and southern *H. lacerata* were distinguishable in multivariate morphological space (MANOVA Wilks' lambda = 0.4576, $df_1 = 8$, $df_2 = 148$, $F = 21.92$, $p < 0.0001$; visualized with PCA in Fig. 5). The first principle component was a gradient from lizards with more connected blotches to lizards with more femoral pores and leg spots. The second principle component was a gradient from lizards with fewer femoral pores to lizards with more connected blotches and leg spots (Table 1). Univariate t-tests showed (after Bonferroni

correction) that northern and southern populations differed significantly in leg length ($t = -4.206$, $p < 0.0001$), side spots ($t = -11.015$, $p < 0.0001$), connected blotches ($t = 3.708$, $p = 0.0004$), femoral pores ($t = 4.6$, $p < 0.0001$) and leg spots ($t = -4.415$, $p < 0.0001$). We also analyzed throat color on 42 male and 39 female northern *H. lacerata* and 26 male and 19 female southern *H. lacerata*. We found that 19% of male and 87% of female northern lizards had orange on their throat while no southern lizards of either sex had any orange throat coloration.

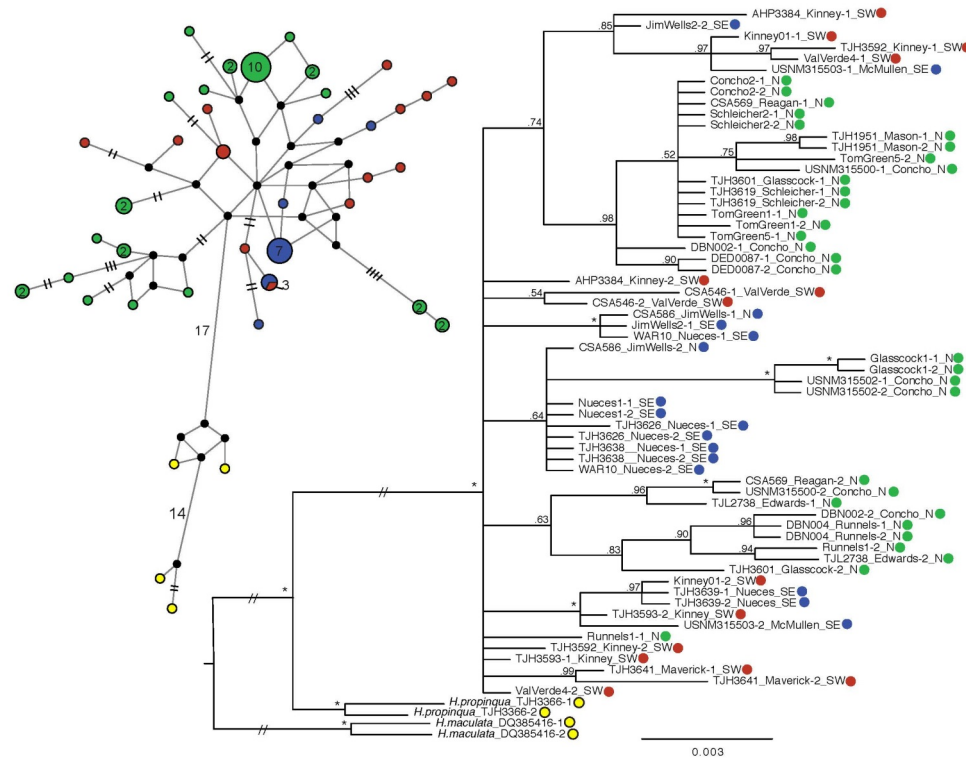


FIGURE 4. Median-joining network (left) of the observed 36 haplotypes for RAG-1 nuclear DNA gene of 66 spotted-tailed earless lizard individuals. Black circles indicate missing intermediates (unsampled). Cross-hatches represent mutational steps with all greater than four denoted by the number of steps. Bayesian analysis of the nuclear DNA RAG-1 gene (right). Numbers at the nodes are posterior probability values. Asterisks indicate values = 1.

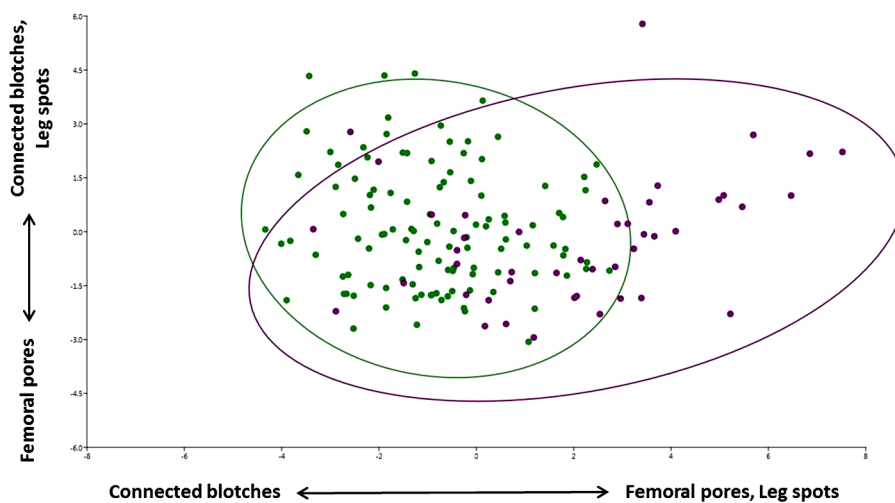


FIGURE 5. Principal components analysis depicting morphometric variables distinguishing northern subspecies, *H. l. lacerata* (green), from southern subspecies, *H. l. subcaudalis* (purple).

TABLE 1. Principle component analysis loadings and percent variance explained on the first four principle components.

Character	PC1	PC2	PC3	PC4
Residual of femur	0.21394	-0.09816	0.044161	0.11725
Residual of humerus	0.028391	-0.00456	0.53391	0.105
Residual head length	-0.01932	0.084818	0.082944	-0.05065
Ventral spots	0.32219	0.086138	-0.11255	0.91769
Dorsal blotches	0.02751	0.022638	0.076205	0.01259
Connected blotches	-0.27386	0.74872	0.46545	0.03281
Left femoral pores	0.53315	-0.34184	0.45341	-0.20979
Leg spots	0.69946	0.54569	-0.26898	-0.29198
% variance explained	35.196	18.298	12.921	11.62

Ecological Niche Correlates. *Holbrookia l. lacerata* had a training AUC of 0.91 and a testing AUC of 0.81 and *H. l. subcaudalis* had a training AUC of 0.81 and a testing AUC of 0.79. These values indicate that the models are informative. The subspecies differ in the climate variables that contribute most to their ecological niche models and projected distributions (Table 2). Mean annual temperature is the most important variable to determine species occurrence for *H. l. lacerata* and precipitation seasonality is the most important variable for *H. l. subcaudalis* (both variables contribute more than 50% of the variation in their respective subspecies). Difference in variable importance contributes to differences in projected geographic distributions of each subspecies (Fig. 6).

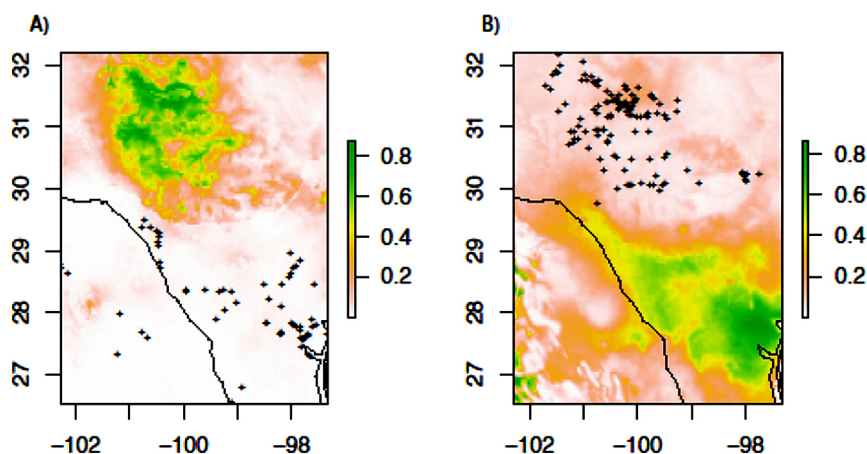


FIGURE 6. Projections for Maxent models into geographic space. The southwestern border between Texas and Mexico is shown as a black segmented line. The color gradient in each panel is identical, ranges from 0 to 0.8, and shows the raw Maxent values (higher values indicate higher suitability for each population). A) Projection of the Maxent model for *Holbrookia lacerata lacerata* shows there is no suitable habitat where *H. l. subcaudalis* occur. *Holbrookia l. subcaudalis* are indicated with the black plus sign. B) Projection of the Maxent model for the *H. l. subcaudalis* shows there is little suitable habitat where *H. l. lacerata* occur. *Holbrookia l. lacerata* are indicated with the black plus sign.

TABLE 2. Variable contribution (%) and permutation importance for each variable to Maxent models for northern and southern populations.

Variable	Variable Contribution		Permutation Importance	
	<i>lacerata</i>	<i>subcaudalis</i>	<i>lacerata</i>	<i>subcaudalis</i>
Mean Annual Temperature	53.99	21.19	55.30	21.17
Annual Precipitation	23.39	13.35	20.30	28.34
Isothermality	10.10	5.09	11.84	18.83
Precipitation Seasonality	6.67	54.01	6.16	28.94
Max Temperature Warmest Month	5.84	6.37	6.41	2.72

Modeled niches were not equivalent ($p < 0.001$), nor were they more similar than expected from background similarity ($p = 0.23$). Tests for niche equivalency showed that the two subspecies were not equivalent (i.e., niches were not identical) using both the D and I metrics for niche equivalency (Fig. 7A). Estimated niche overlap between subspecies was likely explained by regional differences in available habitat (Fig. 7B). The background similarity test was two-tailed, so the results indicate that the overlap was neither more similar than expected (niche conservatism) nor more divergent than expected (niche evolution) given the distribution of background climate variables.

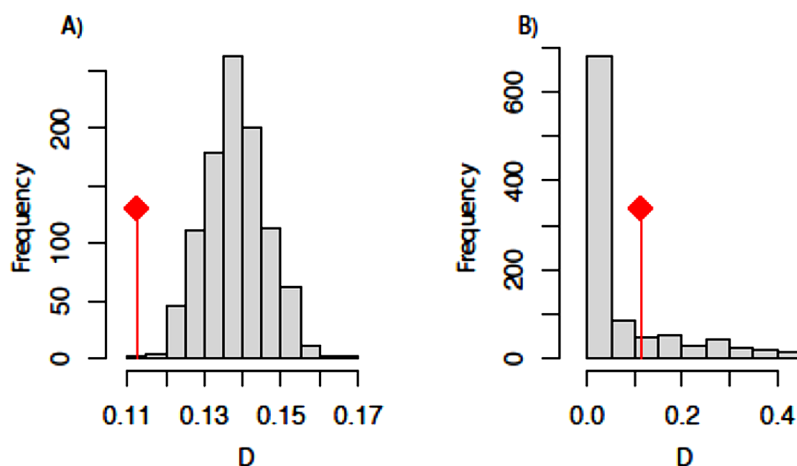


FIGURE 7. Histograms showing D statistic from randomization tests for niche equivalency (A) and background similarity (B). Red diamond pointer shows D statistic position along x-axis.

Species accounts

Holbrookia lacerata Cope 1880

Plateau Spot-tailed Earless Lizard
(Fig. 8)

Holbrookia maculata lacerata Stejneger 1890

Holbrookia lacerata lacerata Axtell 1956

Lectotype. U. S. National Museum (USNM 10160A); collected by G.W. Marnock in May 1879 within a 3 mile radius circle around Helotes, Bexar County, Texas, USA (29° 35' N; 98° 41' W).

Etymology. *Holbrookia* is derived from the last name of the American zoologist John Edwards Holbrook. *Lacerata* is derived from the latin word *lacerare*, which means to cut, destroy, or mangle. Cope (1880) described the posterior border of the transverse brown bars on the dorsum as serrate or digitate. This feature likely brought about the name *lacerata*.

Distribution: *Holbrookia lacerata* includes all populations north of the Balcones Escarpment in Texas. This distribution extends north to the Colorado River, east to the eastern edge of the Balcones Escarpment and west to the Pecos River and southern edge of the Llano Estacado.

Diagnosis. Morphological description based on measurements and counts from 112 adults. This is a small, earless lizard with an average of 4 (0–10) black spots on the underside of the tail. The average snout-vent length (SVL) is 54 mm (32–63), paravertebral and dorsolateral body blotches are often fused. An average of 2 (0–6) blotches are fused out of an average of 6 (4–9) blotches. The blotches form two rows of transverse bands with the dorsal edges of the bands usually narrowing and extending anteriorly. The dark blotches on the hind legs usually form distinct bands

with the average number of leg bands and blotches being 7 (4–11). Some individuals have black lateral spots on the abdomen and these average 0.4 (0–4). The average number of femoral pores on the left leg is 13 (10–17). Females and some males develop a red-orange pattern on their throat and neck during the breeding season.



FIGURE 8. Dorsal (top) and ventral (bottom) views of Plateau Spot-tailed Earless Lizard (*Holbrookia lacerata*) lectotype specimen collected by G.W. Marnock in May 1879 and housed at Smithsonian (USNM 10160).

***Holbrookia subcaudalis* (Axtell 1956)**
Tamaulipan Spot-tailed Earless Lizard
(Fig. 9)

Holbrookia lacerata subcaudalis Axtell 1956
Holbrookia subcaudalis Hibbitts *et al.* 2019 (this study)

Holotype. Texas Natural History Collection, University of Texas (TNHC 20000); collected by Ralph W. Axtell on

6 June 1955 in plowed field 4.8 miles east northeast of Bishop, Nueces County, Texas, USA (27° 36' N; 97° 45' W) at an elevation of 75 feet.

Etymology. The name *subcaudalis* is derived from the latin word *sub* which means under or below and *cauda* which means tail. This refers to the dark spots on the underside of the tail in this species.

Distribution. *Holbrookia subcaudalis* is composed of all populations south of the Balcones Escarpment in Texas and west to the Sierra Madre Oriental in Coahuila, Nuevo Leon, and Tamaulipas, Mexico. They are absent from areas with sandy soils.

Diagnosis. Morphological description based on measurements and counts from 45 adults. This is a small, earless lizard with an average of 5 (1–10) black spots on the underside of the tail. The average snout-vent length (SVL) is 56 mm (31–72), paravertebral and dorsolateral body blotches are often separated. An average of 0.7 (0–6) blotches are fused out of an average of 6 (4–9) blotches. The blotches form four rows of transverse mostly circular blotches. The dark blotches on the rear legs are circular in shape and do not form into bands with the average number of leg blotches being 8 (4–14). Most individuals have black lateral spots on the abdomen and these average 2.6 (0–5). The average number of femoral pores on the left leg is 14 (10–19). The female body color is greenish yellow during the breeding season but they do not acquire orange on the throat in either sex.



FIGURE 9. Dorsal (top) and ventral (bottom) views of Tamaulipan Spot-tailed Earless Lizard (*Holbrookia subcaudalis*) holotype specimen collected by Ralph W. Axtell on 6 June 1955 and housed at Texas Natural History Collection, University of Texas (TNHC 20000).

Discussion

Systematics conclusions. Our genetic BI and ML analyses of the ND2 gene (Fig. 3) demonstrated that *Holbrookia lacerata* encompasses three strongly supported, reciprocally monophyletic lineages, each of which contained individuals from a single geographic region. Our concatenated ND2 and RAG-1 analysis reflected the same geographic lineages, with equally strong levels of support (Supplemental). Our RAG-1 BI and ML analysis did not however show this same structuring (Fig. 4), which indicates that this gene has not fully sorted by lineage and geography. Despite not finding reciprocally monophyletic lineages by geographic region for RAG-1, sub-structuring was clear in the phylogeny and haplotype network suggest that haplotypes were predominately sorted by region. Indeed, we found just one haplotype that was shared between regions, being found in two individuals from Kinney County in the southwest and in two individuals from Nueces County in the southeast (Fig. 4). If the Balcones Escarpment indeed serves as a barrier to gene flow as proposed by Axtell (1968), which is suggested by the ND2 data and morphology, we would expect RAG-1 to sort by geography in the future.

Morphological conclusions. We found that the two species (formerly subspecies) of spot-tailed earless lizards differed significantly in several aspects of their patterns, leg lengths, and in breeding coloration. A MANCOVA also showed that the morphology of the two species differed significantly in multivariate space. In general, *H. subcaudalis* have longer hind limbs, more leg spots, more side spots, fewer connected dorsal blotches, and no orange throat coloration and the opposite is true in *H. lacerata*. Axtell (1956, 1968) observed similar morphological patterns; additionally, he found that *H. subcaudalis* had a larger maximum SVL and a proportionally longer tail than *H. lacerata*.

Ecological niche conclusions. Similar to our other bodies of evidence, the ecological niche of each species divides along the Balcones Escarpment. The most important variable describing *H. lacerata* localities north of this escarpment was mean annual temperature. South of the Balcones Escarpment precipitation seasonality determined the occurrence of *H. subcaudalis*. The Balcones Escarpment is also notable in that it divides two major ecoregions in Texas, the Edwards Plateau and the Southern Texas Plains (Griffith *et al.* 2004).

Taxonomic conclusions. The distinct genetic variation evident in *Holbrookia lacerata* corresponds with the subspecies boundaries (*subcaudalis* and *lacerata*) suggested by Axtell (1968), which were based on morphological differences in body size, pattern, and femoral pore number (Axtell 1956, 1968). The ranges of these subspecies do not overlap, and the southern edge of the Balcones Escarpment forms a zone of broken country that is not suitable habitat for either subspecies. The combined evidence from genetics, morphology and distribution is sufficient to consider these subspecies as distinct species (Wiens & Penkrot 2002) with the lizards north of the Balcones Escarpment retaining the name *Holbrookia lacerata*, while those south of the Balcones Escarpment should be called *Holbrookia subcaudalis*. We were not able to obtain tissue samples from any of the Mexican populations for our genetic analyses; however, based on morphological evidence, these Mexican populations should also be considered *Holbrookia subcaudalis* (Axtell 1968).

No obvious distributional barriers occur between the southeast and southwest populations of *Holbrookia subcaudalis* that can explain the phylogenetic structure observed. Additionally, the only obvious morphological feature that separates the two southern populations is maximum body size, with the largest individuals found in the southeast population (TJH, WAR pers. obs.). The intervening habitat is typical south Texas thornscrub and most of the 11 historical specimens found in the 200 km gap between the two populations were collected between 1950 and 1980. We did not find any additional specimens in this gap during our 2015 survey efforts. Invasive grasses, namely Buffelgrass (*Cenchrus ciliaris*) and King Ranch Bluestem (*Bothriochloa ischaemum* var. *songarica*), and brush encroachment have altered much of south Texas (Archer 1989). Both dense monoculture grasslands and dense south Texas thornscrub are habitats that could negatively affect lizard survival (Scott 1996). These species of grass are widespread in South Texas, and continue to invade more sites since their introduction in the early 1900s (Gould 1975; Tix 2000). Therefore, they may play a role in the apparent reduction in the distribution of *H. subcaudalis*. Closed canopy brushlands are also inhospitable for *H. subcaudalis*, because there is less open space and sunlight available. These lizards are sit-and-wait predators that need open spaces with high visibility for foraging, as well as predator escape and thermoregulation (Hibbitts & Hibbitts 2015).

The Balcones Escarpment limits many other reptile species, notably *Crotaphytus collaris* (Say) and *Diadophis punctatus* (Linnaeus) whose distributions do not extend south of the Balcones Escarpment in Texas. In addition, the distributions of *Drymarchon melanurus* (Duméril, Bibron & Duméril), *Gopherus berlandieri* (Agassiz), and *Scelo-*

porus variabilis Wiegmann do not extend north of the Balcones Escarpment (Smith & Buechner 1947; Dixon 2013). Like *H. lacerata* and *H. subcaudalis*, the sister species *Masticophis taeniatus* (Hallowell) and *M. schotti* (Baird & Girard) also diverge at the Balcones Escarpment (Camper & Dixon 1994).

Conservation Implications. Spot-tailed Earless Lizards are currently under consideration for federal listing as one species (*Holbrookia lacerata*) with two subspecies (*H. l. lacerata* and *H. l. subcaudalis*). The FWS can make decisions on whether to consider the species as a whole or whether one of the two subspecies is in need of endangered species protection. The elevation of these two subspecies to species should require the FWS to consider them separately. The two species have threats that are unique to their regions. *Holbrookia lacerata* is currently thought to be absent from most of the eastern part of its range (Axtell 1998); however, recent observations in Blanco County (iNaturalist 2018), Texas may indicate that isolated pockets persist. The western distribution of *H. lacerata* is much more robust, with *H. lacerata* observed on most surveys in the region (pers. obs.). The situation with *H. subcaudalis* is much different with only two smaller patches of a once larger distribution currently occupied (Fig. 1). The status of the species in Mexico is unknown. The elevation of these taxa makes *H. lacerata* the first endemic lizard in Texas and further highlights the uniqueness of the Edwards Plateau ecoregion in Texas.

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Supplemental. Concatenated Bayesian analysis of ND2 mtDNA and RAG-1 nuclear DNA genes. Numbers at the nodes are posterior probability values. Asterisks indicate values = 1.