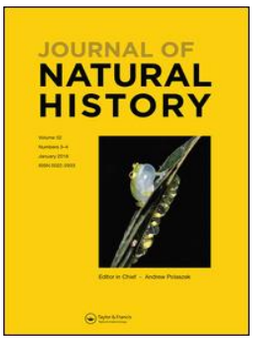

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Mitochondrial genetic variation within and between *Holbrookia lacerata lacerata* and *Holbrookia lacerata subcaudalis*, the spot-tailed earless lizards of Texas

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ABSTRACT

We examined genetic relationships among individuals and populations of the species *Holbrookia lacerata*, the spot-tailed earless lizard, using whole mitochondrial genomes. Lizards were collected from south, central and west Texas. We found significant amounts of genetic structure among populations and evidence of two major reciprocally monophyletic groups of spot-tailed earless lizards in Texas. *Holbrookia lacerata lacerata* occurs on the Edwards Plateau and adjacent regions of West Texas north of the Balcones Escarpment, while *Holbrookia lacerata subcaudalis* occurs in South Texas and adjacent Mexico south of the Balcones Escarpment. These two recognised subspecies correspond to the two clades we discovered. *Holbrookia l. lacerata* occupies much of its historical range at sometimes high population densities, while populations of *H. l. subcaudalis* appear to be highly fragmented based on recent observations compared to their historical range.

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Holbrookia lacerata lacerata; *Holbrookia lacerata subcaudalis*; whole mitochondrial genomes; Texas; Balcones Escarpment; integrative taxonomy

Introduction

Approaches to species delimitation have changed over time with the emergence of new methodologies to quantify and analyse biodiversity. Currently, DNA sequence data are commonplace to identify shared structure in lineages, and thus have naturally been adopted to delimit species based on phylogenetic patterns of gene trees. Recently, the theoretical developments of the multispecies coalescent have provided opportunities to delimit species statistically based on DNA sequence data (Rannala and Yang 2003; Yang and Rannala 2010). Recent controversies have highlighted the limitations of coalescent-based species delimitation and thus the inclusion of additional data types (Sukumaran and Knowles 2017), an approach that has been termed 'integrative taxonomy' (Dayrat 2005;

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Padial et al. 2010). The inclusion of coalescent-based methods with more traditional taxonomic approaches has been advocated as a fruitful approach for species delimitation (Fujita et al. 2012). For this project, we take an integrative taxonomic approach to investigate the lineage independence of *Holbrookia lacerata lacerata* and *Holbrookia lacerata subcaudalis* by using whole mitochondrial genomes, as well as previously published morphological data. The single species previously named the spot-tailed earless lizard (*Holbrookia lacerata*) has two recognised subspecies: *Holbrookia lacerata lacerata* (northern spot-tailed earless lizard) and *Holbrookia lacerata subcaudalis* (southern spot-tailed earless lizard) (Axtell 1956, 1958). Axtell (1956, 1958) published extensively on morphological differences between the two subspecies. *H. l. lacerata* is smaller than *H. l. subcaudalis* (mean snout to vent [SVL] of 52 vs 62 mm, respectively), has fewer femoral pore counts (12.8 vs 15.7 mm, respectively), and differs in meristic characters such as dorsal and leg blotch shape and orientations (Axtell 1956, 1958). *Holbrookia* contains five currently recognised species and, along with the genus *Cophosaurus*, is diagnosed by the lack of a visible auditory meatus. The two genera are part of the 'sand lizard' lineage within the family Phrynosomatidae (Wiens et al. 2010). Morphologically, one fixed character difference exists among both sexes and all ontogenetic age classes of the two spot-tailed earless lizard subspecies. *H. l. lacerata* can be distinguished by rectangular or square-shaped blotches, fused into bands on the hind limbs, while *H. l. subcaudalis* possesses oval or ellipsoid-shaped blotches. While the following are not fixed character differences at all life stages or in all individuals, there are also differences in dorsal blotch shape (fused in *H. l. lacerata* and unfused in *H. l. subcaudalis*), femoral pore counts (approximately four fewer in *H. l. lacerata* vs *H. l. subcaudalis*) and colouration (some female *H. l. lacerata* acquire orange colouration during the breeding season, whereas *H. l. subcaudalis* do not) (Axtell 1956). The two subspecies occur in allopatry, despite occupying similar habitats within their respective ranges. *H. l. lacerata* occurs south and west of the Colorado River on the Edwards Plateau, while *H. l. subcaudalis* occurs across most of south Texas and adjacent Mexico (Figure 1; Axtell 1956).

Methods

To obtain lizard specimens for genetic and morphological examination, we surveyed the museum collection at the University of Texas at Arlington's Amphibian and Reptile Diversity Research Center and collected new specimens from the wild during 2015–2017. Lizards were located by one of two methods: driving roads and looking for live or road-killed individuals, and by walking areas of suitable habitat while visually searching for individuals. Lizards were captured by hand or with the aid of lizard nooses. Surveys were conducted during daylight hours, as *Holbrookia* are diurnal. Sampling effort was concentrated at the warmest time of the day (11:00–16:00 hrs) during the months of March and April. During the warmer months of June–September, survey effort was concentrated in the midmornings (08:00–10:00 hrs) and at dusk (18:00–20:00 hrs) when lizards were most active. If a lizard was found dead, as was common on roads, we collected skeletal muscle, liver, and integumentary tissues and stored them in RNAlater. Live lizards were transported to the lab, where they were euthanised. Tissue samples were collected from skeletal muscle, liver, heart, blood, and integument and stored in RNAlater. Some previously collected

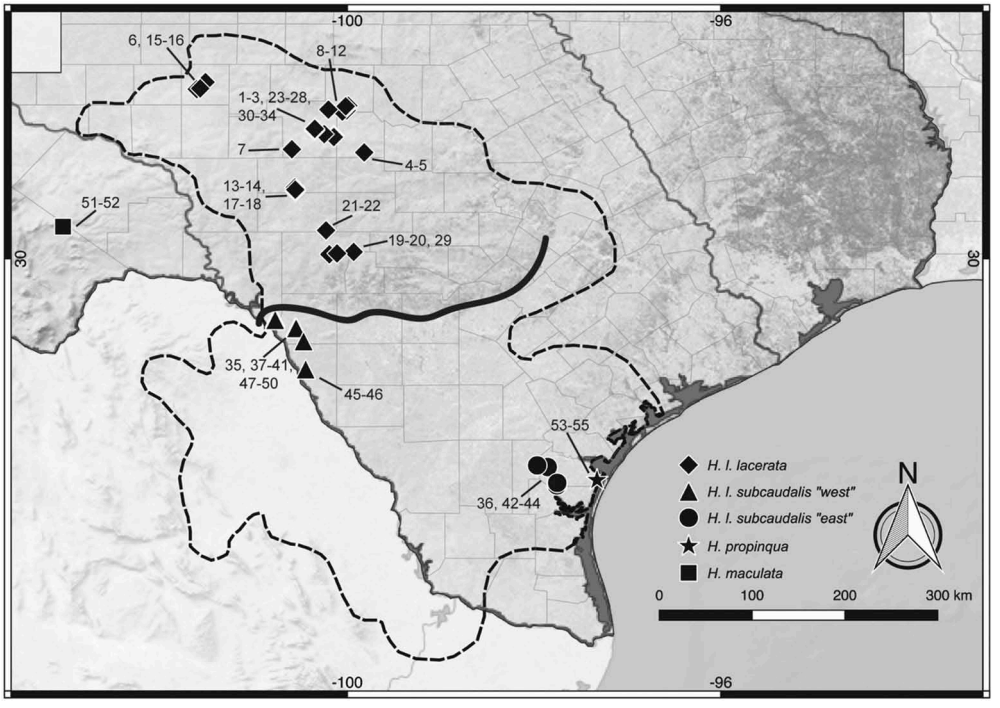

Figure 1. Sampling map of the focal taxa (*Holbrookia lacerata lacerata* and *H. l. subcaudalis*) and outgroup taxa (*H. maculata* and *H. propinqua*). The sampling ranges for the nominal taxa are representative of their current distributions. The historical distribution of *H. lacerata* is represented by the dotted line, while the Balcones Fault/Escarpment, the natural biogeographic barrier between the two subspecies, is represented by the solid black line.

tissues had been stored in ethanol, but that did not influence any laboratory protocols. Additional tissues for this study were obtained from the Biodiversity Research and teaching collections of Texas A&M University and The University of Texas (Appendix 2). We examined a small number of whole specimens of several species of *Holbrookia* (Appendix 1) and counted dorsal blotches, leg blotches and femoral pores.

We extracted DNA from *Holbrookia* tissues stored in ethanol or RNAlater using a standard phenol-chloroform extraction protocol. DNA extractions were quantified on a Qubit 2.0 fluorometer, using the broad range assay kit (Invitrogen). We sequenced the whole mitochondrial genome for *H. l. lacerata* (n = 34), *H. l. subcaudalis* (n = 16), *H. maculata* (n = 2) and *H. propinqua* (n = 3) using the mitochondrial sequencing method developed by the laboratory of Dr Matthew Fujita. Briefly, this protocol first digests the linear nuclear genome using exonucleases, leaving only the circularised mitochondrial genome intact. We amplified the remaining mitochondrial genome using strand-displacement amplification with Φ29 DNA polymerase (NEB). We constructed Illumina libraries from amplified mitochondrial genomes, multiplexing individuals using both inline barcodes and Illumina indices for sequencing on the Illumina HiSeq4000 producing 150-bp paired-end reads.

The Illumina data were processed and cleaned using Fastx-Toolkit v. 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/download.html) and custom Perl scripts. Our adapters

included an 8-bp 'unique molecular identifier' (UMI), which is a random stretch of eight nucleotides at the beginning of each sequenced read. We removed this UMI before demultiplexing individuals based on their unique 5-bp inline barcode. Barcodes and the T-overhang were subsequently removed. We filtered out and discarded low-quality reads if 90% of the nucleotides did not have a Phred score ≥ 20, and the remaining reads were trimmed from both ends if bases had a quality score of ≤ 20. Cleaned reads were assembled using the CLC genomics genome assembler on CLC work bench 7 (Qiagen), producing a ~ 16-kb contig. The assembled whole mitochondrial genomes were annotated on the Mitos Web server to identify the protein-coding, rRNA and tRNA genes (Bernt et al. 2013).

For the phylogenetic analysis, we first used PartitionFinder v. 1.1.1 (Lanfear et al. 2012) to identify the best-supported data partitions (based on the Bayesian information criterion) of the 13 protein-coding genes, separated by codons, from the mitochondrial genome alignments. We found seven partitions with models including HKY (Hasegawa et al. 1985), TrN (Tamura and Nei 1993) and SYM (Zharkikh 1994), some with invariant sites (+I) and some with site variability (+G). We chose to use the HKY+G model in a Bayesian framework to estimate phylogenetic relationships among mitochondrial genomes, rather than more complex models, in order to facilitate convergence during the Markov Chain Monte Carlo (MCMC) run as implemented in BEAST v. 1.8.4 (Drummond et al. 2012). We ran four independent runs, each with 100,000,000 generations, with a burn-in of 10,000,000; all effective sample size (ESS) values for each parameter were 200 for all for runs. As each analysis converged to the same posterior we combined all four analyses into a single posterior to estimate the maximum clade credibility (MCC) tree.

We used the time tree from the BEAST analysis as input for species delimitation using the single-threshold model of the Generalized Mixed Yule Coalescent method (GMYC; Fujisawa and Barraclough 2013). This approach finds the transition from within-species coalescence to between-species (multispecies) coalescence and uses this demarcation as a threshold for delimiting species. The two models tested via GMYC in our data set include whether the samples belong to one species (this includes *lacerata* and the outgroups) or more than one species. We included the outgroups in the GMYC analysis as recommended when focusing on just a few species (in our case, we have one focal taxon, *H. lacerata*; Talavera et al. 2013).

We estimated the maternal effective population sizes of *H. l. lacerata* and *H. l. subcaudalis* using the pairwise distance from whole mitochondrial genomes. This assumes that each subspecies is panmictic, which may be an appropriate assumption for *H. l. lacerata* (which does not have obvious structure based on the phylogeny), but is likely violated for *H. l. subcaudalis* because of its disjunct (and therefore structured) distribution. To determine the effective population size, we equated the average pairwise distance within each subspecies to the population genetic parameter theta (Piganeau and Eyre-Walker 2009). For mitochondrial genomes, theta = 2 × mu × Ne, where mu is the mutation rate per generation. Based on our time-calibrated estimates of mitochondrial mutation rates, we estimate the phrynosomatid lizard substitution rate to be 0.00347×10^{-6} substitutions/site/year. Assuming the equality of mutation and substitution rate (and thus assuming neutral evolution), we set mu = 0.00347×10^{-6} mutation/site/year, or 0.00694 mutations/site/generation assuming a 2-year generation time.

Using average pairwise distances of 0.019 for *H. l. lacerata* and 0.014 for *H. l. subcaudalis*, we can solve for Ne.

Results

We collected 31 individual *H. l. lacerata* and *H. l. subcaudalis* during our surveys (iNaturalist 2017). We also observed another 43 that could not be collected. These lizards were observed in 11 counties. We also collected 18 *H. propinqua* from three counties and 16 *H. maculata* from four counties. All localities for tissues samples used in this study are shown in Figure 1 (GenBank accession numbers MH000136 - MH000189).

The Bayesian phylogenetic analysis of whole mitochondrial genomes yielded a strongly supported topology where *H. l. lacerata* and *H. l. subcaudalis* are reciprocally monophyletic. Sister to the *lacerata* + *subcaudalis* clade is a clade that includes *H. maculata* and *H. propinqua*. The long branches separating each of these four species indicate significant genetic divergence that is a signature of prolonged isolation (Figure 2). Thus, the genetic data support the recognition of *H. l. lacerata* and *H. l. subcaudalis* as distinct subspecies.

The GMYC analysis based on the time tree produced from BEAST identified four potential species in our sampling: provisionally, *H. propinqua*, *H. maculata*, *H. lacerata*

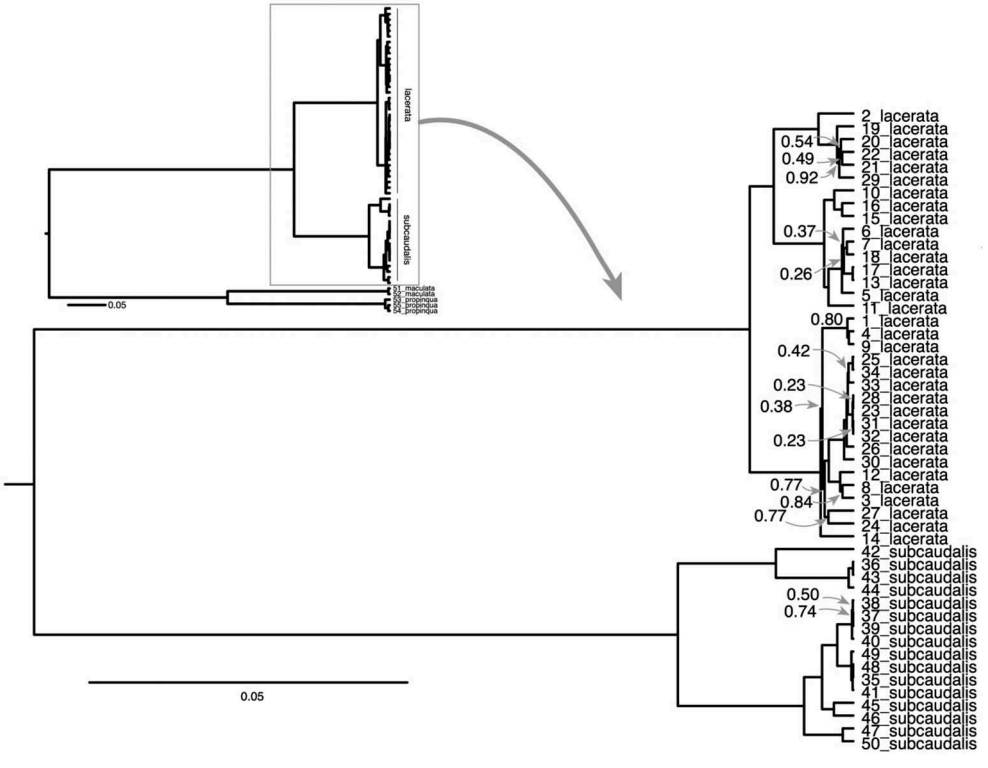

Figure 2. Bayesian phylogeny of whole mitochondrial genomes from *Holbrookia lacerata lacerata* and *H. l. subcaudalis*, with *H. maculata* and *H. propinqua* as outgroup taxa. Numerical values are Bayesian posterior probabilities; all other nodes represent values > 0.95. The scale bar represents percent genetic divergence.

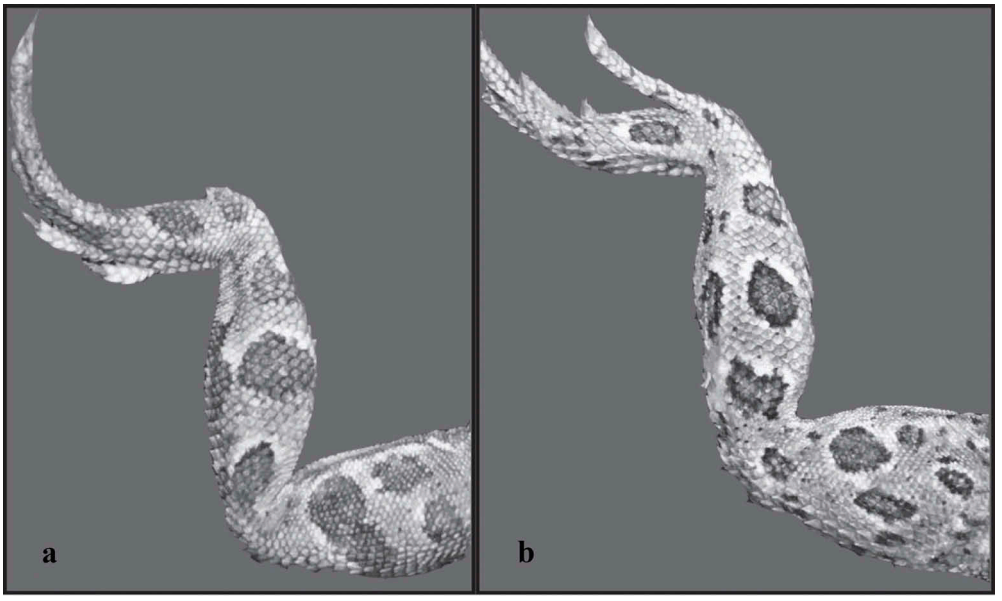

Figure 3. Hind limb blotches of (a) *Holbrookia lacerata lacerata* (UTA R 63333) and (b) *H. l. subcaudalis* (UTA R 63303). In *H. l. lacerata*, most blotches are oblong and fused into bands. In *H. l. subcaudalis*, blotches are ellipsoid.

and *H. subcaudalis*. In our examinations of whole specimens, we noticed no differences in blotch counts or shapes (Figure 3) or femoral pore counts from those reported by Axtell (1956, 1958). These results are consistent with the interpretation that *H. l. lacerata* and *H. l. subcaudalis* are diagnosably distinct (Axtell 1956). When using the estimated mutation rate of 0.00694 mutations/site/generation and pairwise distances estimated from whole mitochondrial genomes, we calculated the maternal effective population size for *H. l. lacerata* to be 1,368,876 individuals and that for *H. l. subcaudalis* to be 1,008,645 individuals.

Discussion

The GMYC analysis supports the recognition of two species of spot-tailed earless lizard clades. Despite some hesitation that GMYC oversplits (Fujisawa and Barraclough 2013), our results suggest it is possible that *H. l. lacerata* and *H. l. subcaudalis* are distinct species. This method identifies the transition between within-species coalescence and between-species coalescence, and uses that threshold to delimit species. One concern with GMYC is that it uses only one locus, and in this case we used the mitochondrial genome which sorts faster and has a higher mutation rate than nuclear loci. Thus, while support for two species based on mitochondrial DNA is strong, that coalescent signal may be less definitive with nuclear markers. Additional data, including genome-wide single nucleotide polymorphisms (SNPs) that are now easier to collect for non-model organisms, analysed using coalescent-based species delimitation tools (BFD*; Leaché et al. 2014) and demographic methods (such as gene flow estimates, e.g. Streicher et al. 2014; Portik et al. forthcoming 2018), can provide deeper insight into the divergence between these two subspecies. As of now, we do not

consider the evidence sufficient to elevate the two subspecies of spot-tailed earless lizard to species.

Effective population sizes are important because they affect population and lineage divergence. We wanted to estimate the effective population sizes of the two subspecies in question to begin understanding their demographic history. While we need additional nuclear data to estimate accurate ancestral effective population sizes and potential gene flow between the two subspecies, our estimates of maternal Ne were quite high for both subspecies, perhaps indicating that the Ne of the ancestral populations was also high. If this is the case, it is likely that the populations of both *H. l. lacerata and H. l. subcaudalis* have been stable despite the deep divergence between the two. While we do not have the data to support this, the long internal branches in the mitochondrial tree indicate substantial divergence that could be habitat-mediated. With additional nuclear data, we should be able to distinguish between selection and nonadaptive forces in the divergence between *H. l. lacerata* and *H. l. subcaudalis*.

Despite the divergence between *H. l. lacerata* and *H. l. subcaudalis*, we did not find significant morphological differences beyond those already described by Axtell (1956). While a more comprehensive morphological assessment is required to identify diagnostic differences between the two subspecies (and perhaps between the distinct mitochondrial genetic clusters within each subspecies), it appears that *H. lacerata* exhibits overall morphological conservatism. These results may support a scenario of divergence in allopatry and the slight morphological differences arose nonadaptively, which could have stemmed from the patchy nature of the lizard's distribution. Axtell (1958) did not believe the slight morphological differences that he used to designate subspecies of *H. lacerata* warranted description of the two forms as full species, and thus their utility as additional evidence for species delimitation may not be satisfactory under an integrative taxonomy framework. Cryptic diversity is a difficult and grey area for species delimitation that relies largely on genetic data, though an integrative taxonomy can incorporate ecology, behavioural and other organismal attributes. Unfortunately, little is known about these for *H. lacerata*, and until additional nuclear sequence data and organismal data become available, it is most prudent to consider the subspecies a single species.

The taxonomic recognition of two diagnosable clades or evolutionarily significant units of spot-tailed earless lizards, currently classified as *H. lacerata*, will have profound effects on the conservation management of the two forms. Currently *H. lacerata* is being treated by the US Fish and Wildlife Service (USFWS) as one species with two subspecies. We believe that based on this paper, *H. l. lacerata* and *H. l. subcaudalis* are discrete entities that warrant consideration for listing by the USFWS under the Endangered Species Act as separate subspecies. Based on this assumption, several conclusions regarding the conservation status of the two subspecies can be made.

The southern spot-tailed earless lizard appears to have undergone substantial reduction in range wide occupancy, leading to two allopatric populations with no geographic intermediates (iNaturalist 2017). Though it remains locally abundant in a small number (< 5) of discrete localities, it is uncommon nearly everywhere else it can still be found within its range. Many) localities where multiple *H. l. subcaudalis* have been found recently (within 5 years) in close geographic proximity are within or immediately adjacent to active grain agricultural fields (iNaturalist 2017).

The northern spot-tailed earless lizard occupies much of its historical range on the Edwards Plateau and Eastern West Texas, based on recent records (iNaturalist 2017), though it appears to have disappeared from many historical localities on the Eastern Edwards Plateau. In some highly human-impacted habitats, most notably fields used for intensive grain agriculture and overgrazed pastures, *H. l. lacerata* can be locally abundant. Sightings of more than 10 individual lizards per hour of observer effort are not uncommon (CER pers. obs.). Unlike *H. l. subcaudalis*, *H. l. lacerata* can be found in many localities devoid of grain agriculture.

Both *H. l. lacerata* and *H. l. subcaudalis* can be abundant in agricultural fields, especially where there are significant proportions of bare soil lacking vegetation. We hypothesise that the tilled soil allows lizards to burrow or exploit burrows made by other animals, and find abundant food in the form of insects, and the large proportions of bare soil and open canopy allow the lizards to easily thermoregulate, engage in social behaviour and forage. We hypothesise that historically, the abundance and range wide occupancy of available habitat could have been positively mediated by the presence of natural fire and grazing of large herbivores, such as American bison (*Bison bison*). Disturbances from these two sources would likely have maintained the open canopy habitats and large areas of bare ground required by both subspecies of spot-tailed earless lizards (Hibbitts and Hibbitts 2015). Assuming lizards can find adequate food and suitable refugia to retreat underground, we believe spot-tailed earless lizards can persist at high population levels in highly human-altered habitats. Historically, many areas in Texas, especially Eastern South Texas, have been exposed to intensive agriculture. We expect this pattern to continue and this should allow at least some subpopulations of both subspecies of spot-tailed earless lizard to maintain healthy population sizes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendices

Appendix 1. Morphological specimens examined

Holbrookia elegans	UTA R 63329
Holbrookia lacerata lacerata	UTA R 32627
Holbrookia lacerata lacerata	UTA R 32641
Holbrookia lacerata lacerata	UTA R 32642
Holbrookia lacerata lacerata	UTA R 38588
Holbrookia lacerata lacerata	UTA R 44012
Holbrookia lacerata lacerata	UTA R 44013
Holbrookia lacerata lacerata	UTA R 55025
Holbrookia lacerata lacerata	UTA R 61067
Holbrookia lacerata lacerata	UTA R 63302
Holbrookia lacerata lacerata	UTA R 63323
Holbrookia lacerata lacerata	UTA R 63324
Holbrookia lacerata lacerata	UTA R 63327
Holbrookia lacerata lacerata	UTA R 63330
Holbrookia lacerata lacerata	UTA R 63331
Holbrookia lacerata lacerata	UTA R 63332
Holbrookia lacerata lacerata	UTA R 63333
Holbrookia lacerata lacerata	UTA R 63334
Holbrookia lacerata lacerata	UTA R 63335
Holbrookia lacerata lacerata	UTA R 63336
Holbrookia lacerata lacerata	UTA R 63337
Holbrookia lacerata lacerata	UTA R 63338
Holbrookia lacerata lacerata	UTA R 63339
Holbrookia lacerata lacerata	UTA R 63340
Holbrookia lacerata subcaudalis	UTA R 57756
Holbrookia lacerata subcaudalis	UTA R 63303
Holbrookia maculata	UTA R 63325
Holbrookia maculata	UTA R 63326
Holbrookia propinqua	CER 200
Holbrookia propinqua	CER 201
Holbrookia propinqua	CER 202
Holbrookia propinqua	CER 937
Holbrookia propinqua	CER 938
Holbrookia propinqua	CER 939
Holbrookia propinqua	CER 940
Holbrookia propinqua	UTA R 37822

Appendix 2. Molecular samples

Field number	Species	Phylogeny number
DED082	*Holbrookia lacerata lacerata*	1_lacerata
DED083	*Holbrookia lacerata lacerata*	2_lacerata
DED084	*Holbrookia lacerata lacerata*	3_lacerata
DED086	*Holbrookia lacerata lacerata*	4_lacerata
DED087	*Holbrookia lacerata lacerata*	5_lacerata
Glasscock 5	*Holbrookia lacerata lacerata*	6_lacerata
MKF854	*Holbrookia lacerata lacerata*	7_lacerata
MKF861	*Holbrookia lacerata lacerata*	8_lacerata
MKF862	*Holbrookia lacerata lacerata*	9_lacerata
Runnels 1	*Holbrookia lacerata lacerata*	10_lacerata
Runnels 2	*Holbrookia lacerata lacerata*	11_lacerata
Runnels 3	*Holbrookia lacerata lacerata*	12_lacerata
Schleicher 1	*Holbrookia lacerata lacerata*	13_lacerata
Schleicher 2	*Holbrookia lacerata lacerata*	14_lacerata
TJH3600	*Holbrookia lacerata lacerata*	15_lacerata
TJH3601	*Holbrookia lacerata lacerata*	16_lacerata
TJH3619	*Holbrookia lacerata lacerata*	17_lacerata
TJH3620	*Holbrookia lacerata lacerata*	18_lacerata
TJH3643	*Holbrookia lacerata lacerata*	19_lacerata
TJH3644	*Holbrookia lacerata lacerata*	20_lacerata
TJH3678	*Holbrookia lacerata lacerata*	21_lacerata
TJH3679	*Holbrookia lacerata lacerata*	22_lacerata
TJH3685	*Holbrookia lacerata lacerata*	23_lacerata
TJH3686	*Holbrookia lacerata lacerata*	24_lacerata
TJH3687	*Holbrookia lacerata lacerata*	25_lacerata
TJH3689	*Holbrookia lacerata lacerata*	27_lacerata
TJH3703	*Holbrookia lacerata lacerata*	28_lacerata
TJL2738	*Holbrookia lacerata lacerata*	29_lacerata
Tom Green 2	*Holbrookia lacerata lacerata*	30_lacerata
Tom Green 4	*Holbrookia lacerata lacerata*	31_lacerata
Tom Green 6	*Holbrookia lacerata lacerata*	32_lacerata
Tom Green 7	*Holbrookia lacerata lacerata*	33_lacerata
Tom Green 8	*Holbrookia lacerata lacerata*	34_lacerata
CSA546	*Holbrookia lacerata subcaudalis*	35_subcaudalis
Jim Wells 2	*Holbrookia lacerata subcaudalis*	36_subcaudalis
Kinney 1	*Holbrookia lacerata subcaudalis*	37_subcaudalis
Kinney 2	*Holbrookia lacerata subcaudalis*	38_subcaudalis
Kinney 3	*Holbrookia lacerata subcaudalis*	39_subcaudalis
Kinney 4	*Holbrookia lacerata subcaudalis*	40_subcaudalis
TJH3588	*Holbrookia lacerata subcaudalis*	41_subcaudalis
TJH3626	*Holbrookia lacerata subcaudalis*	42_subcaudalis
TJH3637	*Holbrookia lacerata subcaudalis*	43_subcaudalis
TJH3638	*Holbrookia lacerata subcaudalis*	44_subcaudalis
TJH3640	*Holbrookia lacerata subcaudalis*	45_subcaudalis
TJH3641	*Holbrookia lacerata subcaudalis*	46_subcaudalis
Val Verde 2	*Holbrookia lacerata subcaudalis*	47_subcaudalis
Val Verde 3	*Holbrookia lacerata subcaudalis*	48_subcaudalis
Val Verde 4	*Holbrookia lacerata subcaudalis*	49_subcaudalis
Val Verde 5	*Holbrookia lacerata subcaudalis*	50_subcaudalis
MKF844	*Holbrookia maculata*	51_maculata
MKF848	*Holbrookia maculata*	52_maculata
CER1065	*Holbrookia propinqua*	53_propinqua
CER1066	*Holbrookia propinqua*	54_propinqua
CER1067	*Holbrookia propinqua*	55_propinqua