Section 6 (Texas Traditional) Report Review

Form emailed to FWS S6 coordinator (mm/dd/yyyy): 9/13/2017

TPWD signature date on report: 8/31/2017

Project Title: Evaluation of the species level status, population genetic structure, and connectivity among populations of the spot tailed earless lizard (*Holbrookia lacerata*).

Final or Interim Report? Final

Grant #: E-174

Reviewer Station: Austin ESFO

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

Interim Report (check one):

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

Final Report (check one):

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

Comments:

FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-174-R

(F15AP00673)

Endangered and Threatened Species Conservation

Evaluation of the species level status, population genetic structure, and connectivity among populations of the spot tailed earless lizard (*Holbrookia lacerata*).

Prepared by:

Corey Roelke and Matthew Fujita

Carter Smith
Executive Director

Clayton Wolf
Director, Wildlife

31 August 2017

FINAL REPORT

STATE: Texas GRANT NUMBER: TX E-174-R-1

GRANT TITLE: Evaluation of the species level status, population genetic structure, and connectivity among populations of the spot tailed earless lizard (*Holbrookia lacerata*).

REPORTING PERIOD: 1 September 2015 to 31 August 2017

OBJECTIVE(S). To provide an ecological, genetic, and species delimitation evaluation for the spot tailed earless lizard (*Holbrookia lacerata*) that will impart insight on the development of its conservation and management.

Segment Objectives:

Task #1: Conduct fieldwork; acquisition of samples: July 2015 – Oct 2015; July 2016 – Oct 2016
Task #2: Develop the exome capture system: July 2015 – August 2015
Task #3: *Holbrookia* phylogeny using exome capture: September 2015-December 2015.
Task #4: Species delimitation using exome capture: January 2016 – April 2016.
Task #5: Demographic inferences using whole mitochondrial genomes and RADseq: Sep 2016 – Dec 2016

Significant Deviations:

None.

Summary Of Progress:

Please see Attachment A.

Location: Range or spot-tailed earless lizard (see Project Statement), Texas, USA.

Cost: Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.

Prepared by: Craig Farquhar Date: 31 August 2017

Approved by:  Date: 31 August 2017
C. Craig Farquhar

ATTACHMENT A

Evaluation of the species level status, population genetic structure, and connectivity among populations of the spot tailed earless lizard (*Holbrookia lacerata*).

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Abstract

We examined genetic relationships among individuals and populations of the species previously recognized as *Holbrookia lacerata*, the Spot-tailed Earless Lizard, using whole mitochondrial genomes and some components of the nuclear genome. Lizards were collected from South, Central, and West Texas. We found significant amounts of genetic structure among populations and recognize the validity of two species of Spot-tailed Earless Lizard in Texas. *Holbrookia lacerata* occurs on the Edwards Plateau and adjacent regions of West Texas North of the Balcones Escarpment, while *Holbrookia subcaudalis* occurs in South Texas and adjacent Mexico South of the Balcones Escarpment. *H. lacerata* occupies much of its historic range at sometimes high population densities, while populations of *H. subcaudalis* are highly fragmented and the species has seen a reduction in range wide habitat occupancy.

Introduction

Approaches to species delimitation have changed over time with the emergence of new methodologies to quantify and interrogate biodiversity. Currently, DNA sequence data are commonplace to identify 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 statistically delimit species 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; 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* and *Holbrookia subcaudalis* by using whole mitochondrial genomes as well as morphological data. The single species previously named the Spot-tailed Earless Lizard (*Holbrookia lacerata*), has recently been split into two full species by the elevation of previously recognized subspecies: the Plateau Spot-tailed earless lizard (*Holbrookia lacerata lacerata*) and the Taumalipan Spot-tailed Earless Lizard (*Holbrookia lacerata subcaudalis*) (Hibbitts et al., in press). One nuclear gene (*RAG-1*), 1054 base pairs (bp) in length, and one mitochondrial gene (*ND2*), 1086 bp in length were sequenced and compared among individuals of the two subspecies. Analysis of these genetic data, patterns of distribution, and previously published morphological data (Axtell 1956, 1958), led the authors to conclude there was ample evidence to elevate both subspecies to specific status.

Both species of Spot-tailed Earless Lizard represent reciprocally monophyletic lineages in the genus *Holbrookia*. *Holbrookia* contains five currently recognized species (Hibbitts et al., in press) defined by the lack of a visible auditory meatus and is 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 species. *H. lacerata* can be distinguished by rectangular or square shaped blotches, fused into bands on the hindlimbs while *H. subcaudalis* possesses oval or ellipsoid shaped blotches. While not fixed character differences at all life stages or in all individuals, there are also differences in dorsal blotch shape: unfused in *H. lacerata* and fused in *H. subcaudalis*, femoral pore counts: approximately four fewer in *H. lacerata* vs. *H subcaudalis*, and coloration: some *H. lacerata* acquire orange coloration during the breeding season whereas *H. subcaudalis* do not (Hibbitts et al., in press, Axtell, 1956, 1958). The two species occur in allopatry, despite occupying similar habitats within their respective ranges. *H. lacerata* occurs North and East of the Colorado River on the Edwards Plateau while *H. subcaudalis* occurs across most of South Texas and adjacent Mexico (Axtell, 1968).

Objective

To provide an ecological, genetic, and species delimitation evaluation for the spot tailed earless lizard (*Holbrookia lacerata*) that will impart insight on the development of its conservation and management.

Methods

To obtain lizard specimens for genetic and morphological analysis, 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 portions of the day (1100–1600 hrs.) during the months of March and April. During the warmer months of June–September, survey effort was concentrated in the midmornings (0800–1000 hrs) and at dusk (1800–2000 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 euthanized. Tissue samples were collected from skeletal muscle, liver, heart, blood, and integument and stored in RNAlater. Some previously collected tissues had been stored in ethanol, but that did not influence any laboratory protocols. Some tissues for this study were obtained from the tissue collections of Texas A&M University and The University of Texas.

We extracted DNA from *Holbrookia* tissues stored in ethanol or RNAlater or ethanol 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. lacerata* (n=34), *H. 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 circularized 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 150bp paired-end reads.

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

We collected our first ddRADseq data for 103 individuals. The data were processed as in Streicher et al. (2014). For these preliminary data, we chose to focus on 17 individuals with the most complete datasets (12 Holbrookia lacerata and 5 H. subcaudalis). Based on this dataset, we recovered 7,342 loci (17,763 single nucleotide polymorphisms or SNPs) for H. lacerata and 4,970 loci (13,413 SNPs) for H. subcaudalis. When we analyzed each species independently, we found that H. lacerata and

subcaudalis differed in their heterozygosity (0.124 vs. 0.140, respectively), indicating that they each have unique demographic histories. We are still in the process of obtaining additional ddRADseq data that will provide more complete data for the other individuals and allow us the measure the extent of gene flow between these two species.

Results

We observed and collected thirty one (31) individual *H. lacerata* and *H. subcaudalis* during our surveys (iNaturalist, 2017). We also observed another forty three (43) that could not be collected. These lizards were observed in eleven counties. We also collected eighteen (18) *H. propinqua* from three counties. We collected sixteen (16) *H. maculata* from four counties.

The Bayesian phylogenetic analysis of whole mitochondrial genomes yielded a strongly-supported topology where *Holbrookia lacerata* and *H. subcaudalis* are reciprocally monophyletic. Sister to the *lacerata*+*subcaudalis* is a clade that includes *H. maculata* and *H. propinqua*. The long branches separated each of these four species indicates significant genetic divergence that is a signature of prolonged isolation. Thus, the genetic data support the recognizition of *H. lacerata* and *H. subcaudalis* as distinct species.

Discussion

The taxonomic recognition of two sister species of Spot-tailed earless lizards, both previously considered *H. lacerata*, will have profound effects on the conservation management of the two species. 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 the recent taxonomic revision by Hibbitts et al. (in press) and this paper, *H. lacerata* and *H. subcaudalis* will receive consideration for listing by the USFWS under the Endangered Species Act as separate species. Based on this assumption, several conclusions regarding the conservation status of the two species can be made.

The Taumalipan Spot-tailed Earless Lizard appears to have undergone substantial reduction in range wide occupancy, leading to two allopatric populations with no geographic intermediates (Hibbitts et al., in press). 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. Nearly all recent (within five years) localities where multiple *H. subcadualis* have been found in close geographic proximity are within or immediately adjacent to active grain agricultural fields (iNaturalist, 2017).

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

Both *H. lacerata* and *H. subcaudalis* can be abundant in agricultural fields, especially where there are significant proportions of bare soil lacking vegetation. We hypothesize that the tilled soil allows lizards to easily burrow or exploit burrows made by other animals, 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 behavior, and forage. We hypothesize that historically, the abundance and range wide occupancy of available habitat was 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 species 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 species of Spot-tailed earless lizard to maintain healthy population sizes.

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Acknowledgements

We thank the biologists of the Texas Parks and Wildlife Department. We thank the many landowners and land managers, both public and private, who granted us access to their land for lizard surveys. This work was funded by a US Fish & Wildlife Service Section 6 Grant (TX E-174-R) administered by the Texas Parks and Wildlife Department awarded to CER and MKF. Research on this project was approved by the University of Texas at Arlington's IACUC protocol #A16.010

Figures

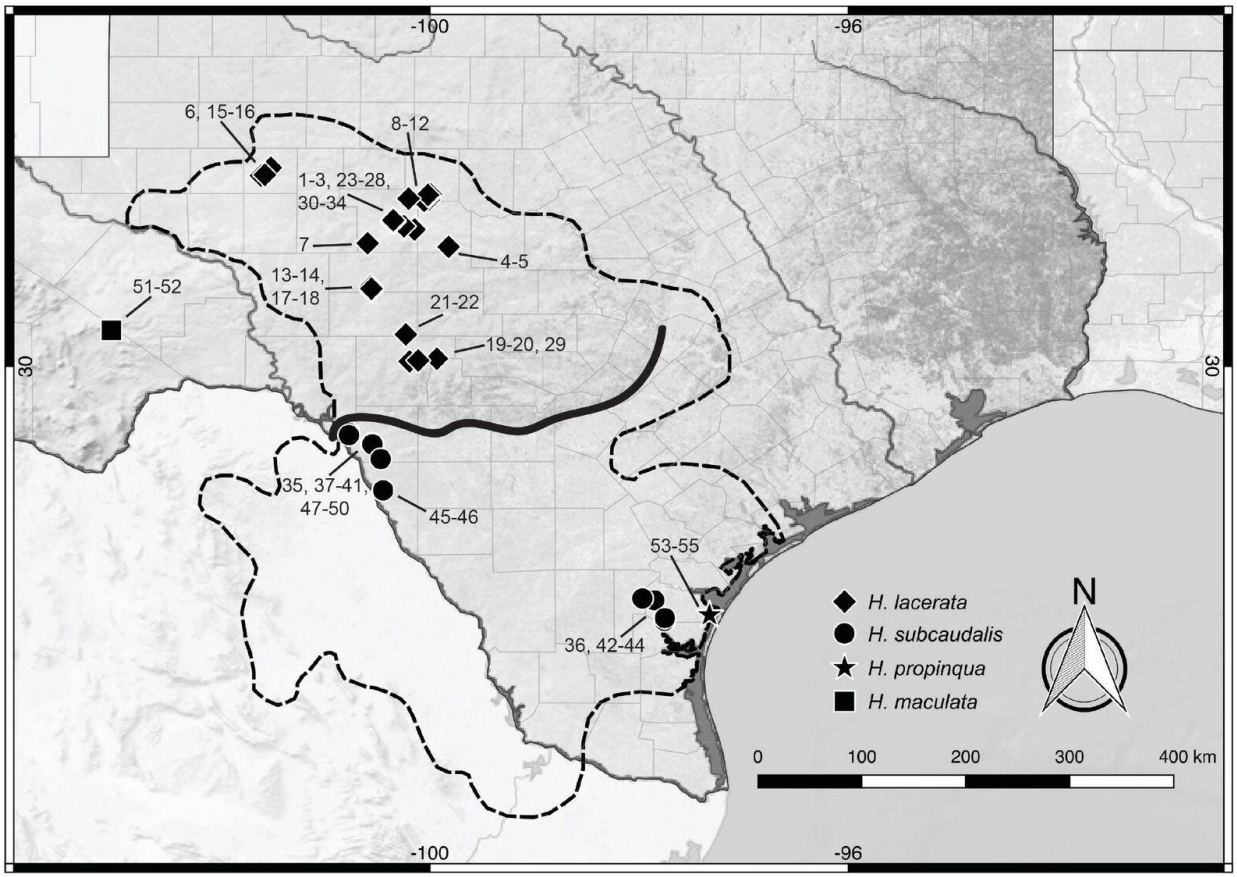

Figure 1. Map of localities sequenced for whole mitochondrial genomes of the four species of Texas *Holbrookia.* Sample numbers correspond to individual specimens labelled in Figure 2.

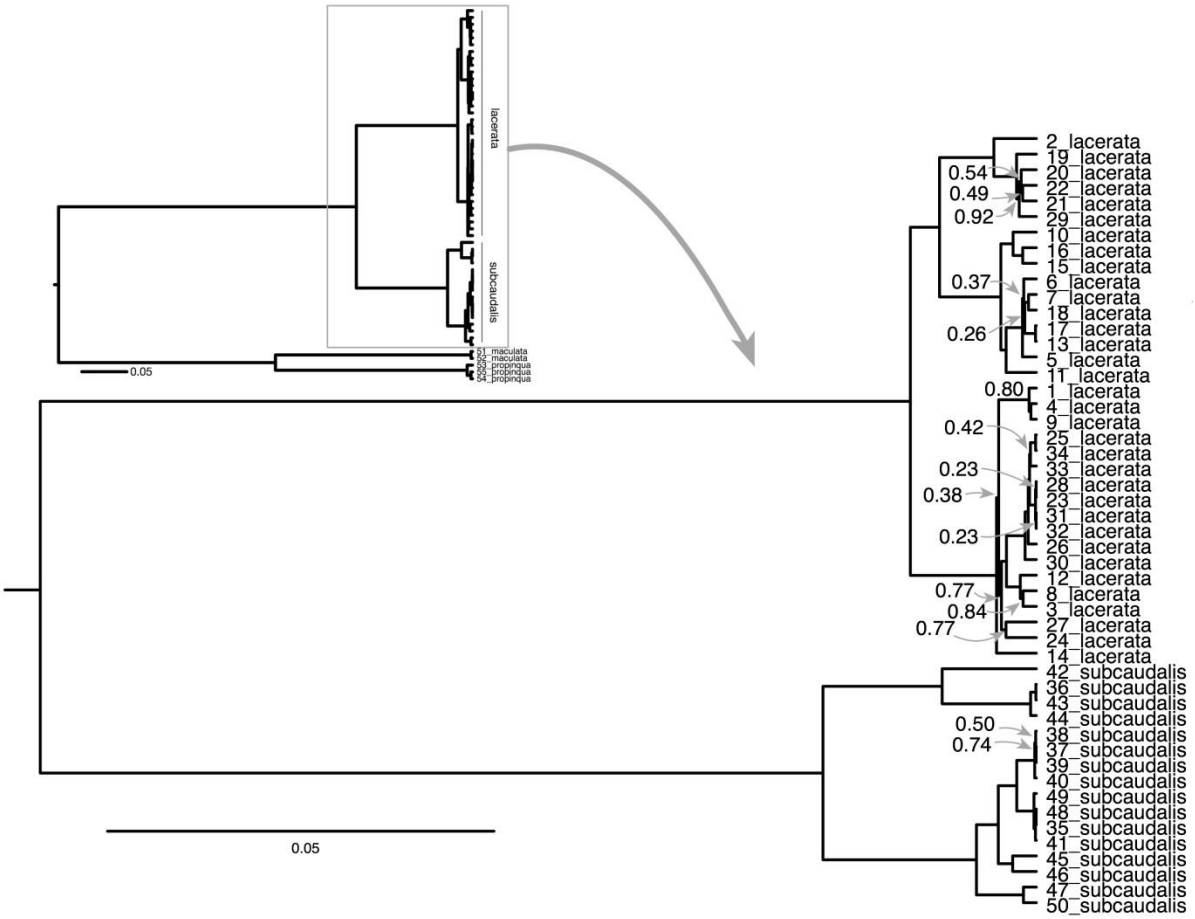

Figure 2. Bayesian phylogeny of whole mitochondrial genomes from *H. lacerata* and *H. subcaudalis*, with *H. maculata* and *H. propinqua* as outgroup taxa. Numerical values are Bayesian posterior probabilities; all other nodes represent values > 0.95. Scale bar represents percent genetic divergence.

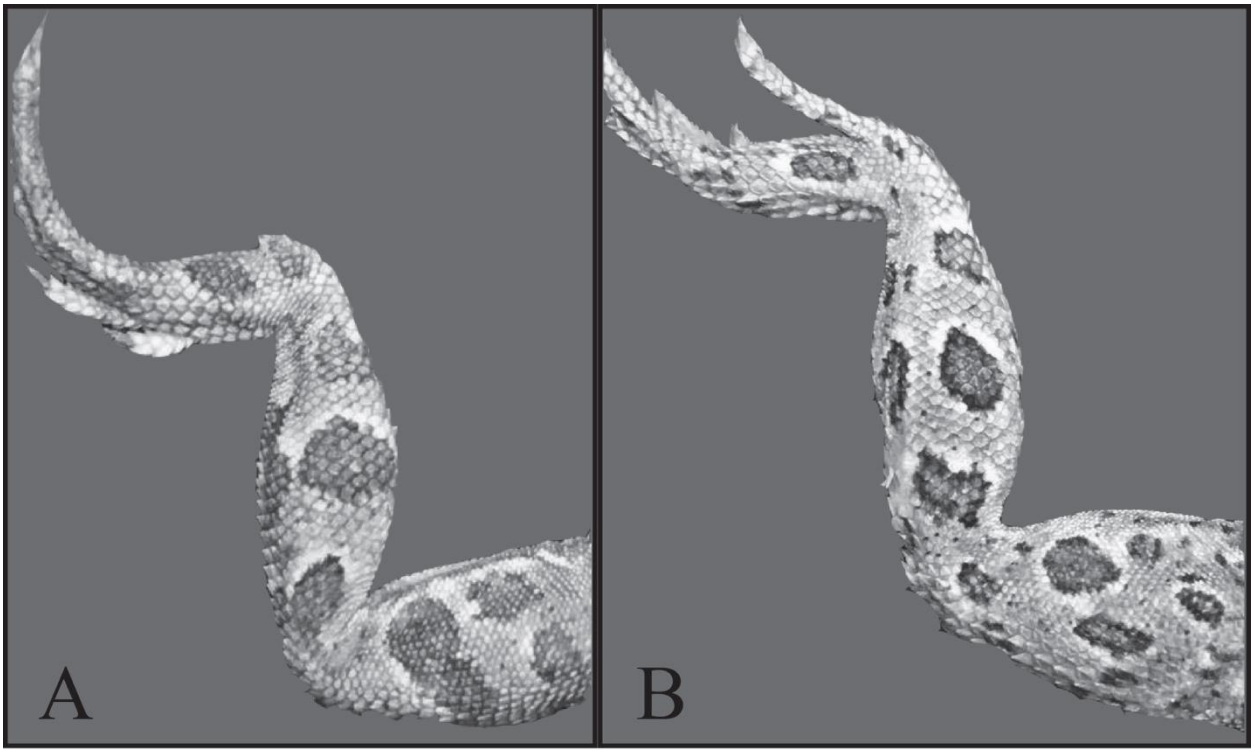

Figure 3. Hindlimb blotches of A) *Holbrookia lacerata* (UTA R 63333) and B) *H. subcaudalis* (UTA R 63303). In *H. lacerata*, most blotches are oblong and fused into bands. In *H. subcaudalis*, blotches are ellipsoid.

Appendix – Morphological specimens examined

Holbrookia elegans	UTA R 63329
Holbrookia lacerata	UTA R 32627
Holbrookia lacerata	UTA R 32641
Holbrookia lacerata	UTA R 32642
Holbrookia lacerata	UTA R 38588
Holbrookia lacerata	UTA R 44012
Holbrookia lacerata	UTA R 44013

Holbrookia lacerata	UTA R 55025
Holbrookia lacerata	UTAR 61067
Holbrookia lacerata	UTA R 63302
Holbrookia lacerata	UTA R 63303
Holbrookia lacerata	UTA R 63323
Holbrookia lacerata	UTA R 63324
Holbrookia lacerata	UTA R 63327
Holbrookia lacerata	UTA R 63330
Holbrookia lacerata	UTA R 63331
Holbrookia lacerata	UTA R 63332
Holbrookia lacerata	UTA R 63333
Holbrookia lacerata	UTA R 63334
Holbrookia lacerata	UTA R 63335
Holbrookia lacerata	UTA R 63336
Holbrookia lacerata	UTA R 63337
Holbrookia lacerata	UTA R 63338
Holbrookia lacerata	UTA R 63339
Holbrookia lacerata	UTA R 63340
Holbrookia maculata	UTA R 44249
Holbrookia maculata	UTA R 44250
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
Holbrookia subcaudalis	UTA R 57756
Holbrookia subcaudalis	UTA R 63303

Significant deviations

We have acquired all of the materials to pursue the sequence capture of the transcriptome-based genes. These experiments will be conducted within the next month, and we anticipate submitting a publication about the genus-wide phylogeny and species delimitation by December 2017.