5-2018

Regional Distribution, Non-invasive Detection, and Genetic Diversity of the Black-spotted Newt (Notophthalmus meridionalis)

Evan A. Bare
The University of Texas Rio Grande Valley

REGIONAL DISTRIBUTION, NON-INVASIVE DETECTION, AND GENETIC DIVERSITY OF THE BLACK-SPOTTED NEWT (*NOTOPHTHALMUS MERIDIONALIS*)

A Thesis

by

EVAN A. BARE

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2018

Major Subject: Biology

REGIONAL DISTRIBUTION, NON-INVASIVE DETECTION, AND GENETIC DIVERSITY OF THE BLACK-SPOTTED NEWT (*NOTOPHTHALMUS MERIDIONALIS*)

A Thesis
by
EVAN A. BARE

COMMITTEE MEMBERS

Dr. Richard Kline
Chair of Committee

Dr. Diego Figueroa
Committee Member

Dr. Andy Gluesenkamp
Committee Member

May 2018

ABSTRACT

Bare, Evan A., Regional Distribution, Non-Invasive Detection, and Population Genetics of the Black-Spotted Newt (*Notophthalmus meridionalis*). Master of Science (MS), May, 2018, 95 pp., 17 tables, 27 figures, references 231 titles.

The Black-spotted Newt (*Notophthalmus meridionalis*) is one of three a salamander species native to Gulf Coast prairies of Texas and Mexico, with respective state and federal protections. This species has been neglected by the scientific community despite concerns of dramatic population declines and a globally endangered status, with the most recent work being conducted in the early 1990's going unpublished. This study presents the most recent examination of the species providing probabilistic distribution maps, descriptions of three novel populations, assessments of surveying techniques, and the first known examination of intrapopulation genetics including the first documented genetic examination of the southern subspecies. An updated status review is provided in response to the results of this study and an expansive literature review.

DEDICATION

To my father, for instilling a fascination and appreciation for science, and mother, without whose continual and persistent dedication to my education I can never fully repay.

ACKNOWLEDGEMENTS

I must pay thanks to my committee chair and adviser Dr. Richard Kline for allowing me the opportunity to work on this unique species and for all his guidance through my graduate studies. I am tremendously grateful to my roommates Al Alder and Caleb Arellano for providing constant support and advice. I would also like to thank Dr. Abdullah Rahman and Dr. Daniele Provenzano for teaching me proper genetic protocols and offering suggestions. Thanks as well to the Texas Parks and Wildlife Department and the Southwest Center for Herpetological Research for funding this project. Lastly, to all my lab mates and field hands that have helped over the years of this project – thank you all.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Amphibian populations across the globe have been in steady decline during the 21st century, with nearly 41% of amphibian species considered threatened or endangered as of 2015 (IUCN, 2015), and are projected to decline in the near future (Hof, Araújo, Jetz, & Rahbek, 2011; Raffel, Rohr, Kiesecker, & Hudson, 2006; Stuart SN, Chanson JS, Cox NA, Young BE & Fischman DL, 2004). Disease (Group et al., 2016; Hof et al., 2011; Hoverman, Gray, Miller, & Haislip, 2012; Laking, Ngo, Pasmans, Martel, & Nguyen, 2017), climate change (Raffel et al., 2006), habitat fragmentation (Cushman, 2006; Ficetola, Rondinini, Bonardi, Baisero, & Padoa-Schioppa, 2015; Johansson, Primmer, & Merilä, 2007; Joly, Morand, & Cohas, 2003), and human activity (Brum et al., 2013; Cosentino et al., 2014; Glista, DeVault, & DeWoody, 2008; Langen, Ogden, & Schwarting, 2009) have all been pointed to as drivers for this decline. Consequentially, ecosystems have been losing natural modes of energy transfer (Gibbons et al., 2006) from aquatic to terrestrial environments and key indicator species (Tiago, Pereira, & Capinha, 2017; Waddle, 2006; Wilson & McCranie, 2003). While amphibian species discoveries have continued in recent decades (Frank & Kohler, 1998; Hanken, 1999; Köhler et al., 2005), many are listed immediately after discovery due to data deficiencies (IUCN, 2015), begging the question: how many are lost before being discovered?

Salamanders comprise 9.25% of amphibian species (Frost 2017) with roughly 20% being native to the United States (Stein, Kutner, & Adams, 2000). Even though there are just over 700

species world-wide (Frost, 2017), salamanders inhabit a wide diversity of habitats ranging from the fossorial Ambystomids, to cave-dwelling *Eurycea* species, and arboreal *Aneides* (Conant & Collins, 1998). Ecosystems are greatly influenced by salamanders, demonstrating significant influence on invertebrate populations (Best & Welsh, Jr., 2014; Petranka, 1998; Walton, 2013; Walton, Tsatiris, & Rivera-Sostre, 2006), leaf litter retention (Wyman, 1998), and other amphibian assemblages (Fauth, Resetarits, & Resetarits, 1991; Werner & McPeek, 1994; Wilbur, Morin, & Harris, 1983). In addition, salamanders are able to sequester vast amounts of nutrients and energy from an ecosystem, even surpassing that of mammals and birds during peak season (Burton & Likens, 1975a, 1975b; Semlitsch, O'Donnell, & Thompson III, 2014). Often considered indicator species (Waddle, 2006; Wilson & McCranie, 2003), salamander presence has been linked to water quality (Bodinof Jachowski, Millspaugh, & Hopkins, 2016; Freda, 1991; Robinson, 1993), soil quality (Malcolm Pratt Frisbie & Wyman, 1991; M P Frisbie & Wyman, 1992; Wyman, 1988; Wyman & Jancola, 1992), and forest successional stage (Hicks & Pearson, 2003; Pittman & Semlitsch, 2013),

Only three salamander species are known to share habitats between the United States and Mexico (Babb et al., 2015), each of which is native to the Lower Rio Grande Valley (LRGV) (Conant & Collins, 1998): the Barred Tiger Salamander (*Ambystoma tigrinum mavortium*), the Rio Grande Lesser Siren (*Siren intermedia texana*), and the Black-spotted Newt (*Notophthalmus meridionalis meridionalis*). These salamanders will favor life underground during dry conditions that frequent the LRGV(Conant & Collins, 1998) . Due to their unique habitat and rarity, very little scientific focus has been given to these species, especially the Black-spotted Newt.

The Black-spotted Newt (BSN) is a small-medium sized salamander with stocky build and a total length generally between 71-110 mm (Mecham, 1968a). Like other *Notophthalmus* species

(Mecham 1967ab), BSN has an olive green to brown ground color and yellow-orange venter with black-spots speckled throughout the body. Dorsal yellow to golden lateral bars and the eponymous large black spots are distinguishing markers (Figure 1). First identified by Edward D. Cope (1880) as a subspecies of *Diemyctylus miniatus*, there are two recognized subspecies of BSN (Mecham 1968ab): the Texas Black-spotted Newt (Crother, 2012), *N. m. meridionalis*; and the Mexico Black-spotted Newt (Liner & Casas-Andreu, 2008), *N. m. kallerti*. Mecham (1968ab) described a zone of intergradation centered around 22.5° N latitude in Tamaulipas, Mexico with *N. m. meridionalis* to the north and *N. m. kallerti* to the south (Figure 2). The two subspecies have been distinguished by morphological characteristics (Mecham 1968ab), but no genetic or ecological comparison between these two subspecies has been conducted.

The Black-spotted Newt is the only salamander species considered exclusively endemic to the Tamaulipan Biotic Province of Texas and Mexico (Babb et al., 2015;

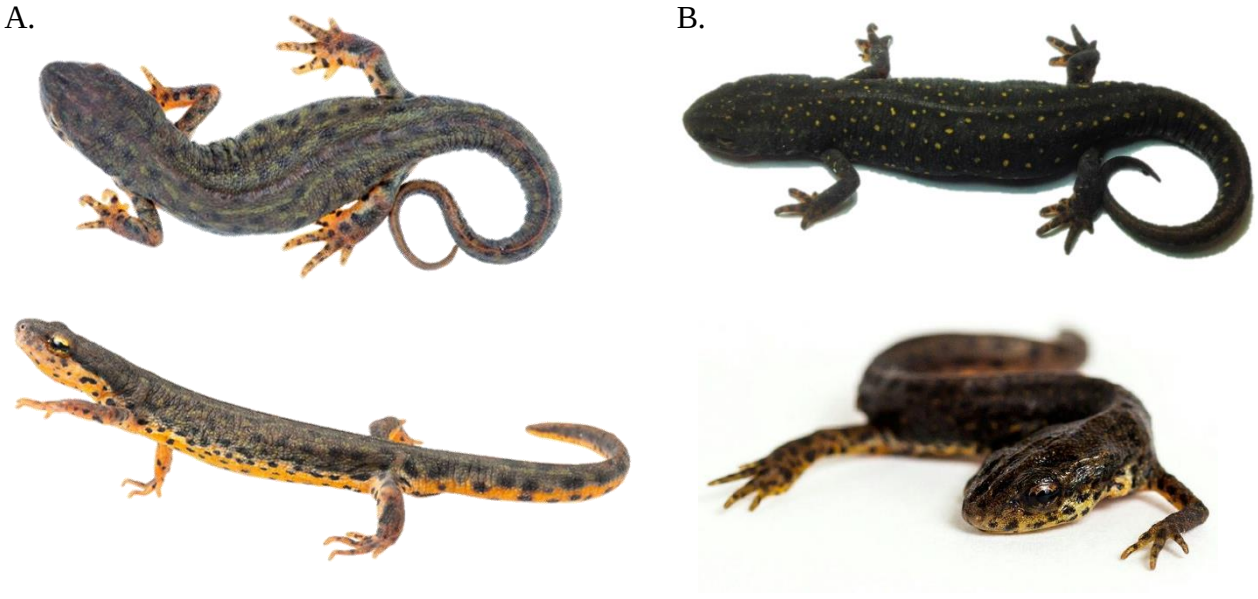

Figure 1. Dorsal and profile views of *N. m .meridionalis* (A) and *N. m. kallerti* (B). Photos by Seth Patterson (A) and Jaime Peña (B)

Rappole & Klicka, 1991) and is listed as the only globally endangered amphibian along the US-Mexico border in a cross country examination (Young & Sanchez, 2006). This salamander resides along the coastal plains bordering the Gulf of Mexico: ranging north to San Patricio County, Texas down south to central Veracruz, Mexico (Judd 1985; Mecham 1968ab; Rappole and Klicka 1991; see Figure 1). Conservation groups have recognized the species as Globally Endangered (IUCN), while it is listed in Mexico as "en peligro de extincíon" (NOM-059-SEMARNAT-2010), and Threatened in the state of Texas (TPWD). In 2007, the Forest Guardians and WildEarth Guardians petitioned the EPA to list the species as Endangered under the Endangered Species Act (Rosmarino & Tutchton, 2007), listing their critically imperiled (G1) status by NatureServe and claiming there to be only two known populations in Texas and one in Mexico (WildEarth Guardians, 2010), likely referencing Judd (1985).

Within the Tamaulipan Biotic Province, the species is primarily restricted to a 30 -50 km wide region along the coastal plain (Rappole & Klicka, 1991) but have been identified farther inland (Carbajal-Marquez, Quintero-Diaz, & De la Vega, 2014; Mecham, 1968a; Taggart, 1997). Mexican specimens have been found at altitudes up to 800 m in the Sierra de Tamaulipas and the Sierra Madre (Mecham, 1968a), but are generally restricted to low lying wetlands. Typically found in the vicinity of resacas (historic channels of the Rio Grande) or ephemeral ponds (Mecham, 1968a), black-spotted newts are associated with thick vegetation, especially *Chara* algae, but have also been described as residents of " lagoons, and swampy areas" (Conant & Collins, 1998) of the coastal plains, scarcities in the semi-arid region.

This study was conducted within the Lower Rio Grande Valley (LRGV) of Texas, which is comprised of four counties: Cameron, Willacy, Hidalgo, and Starr. The LRGV is one the most rapidly developing areas of the US (Jahrsdoerfer & Leslie, 1988) with human populations greatly

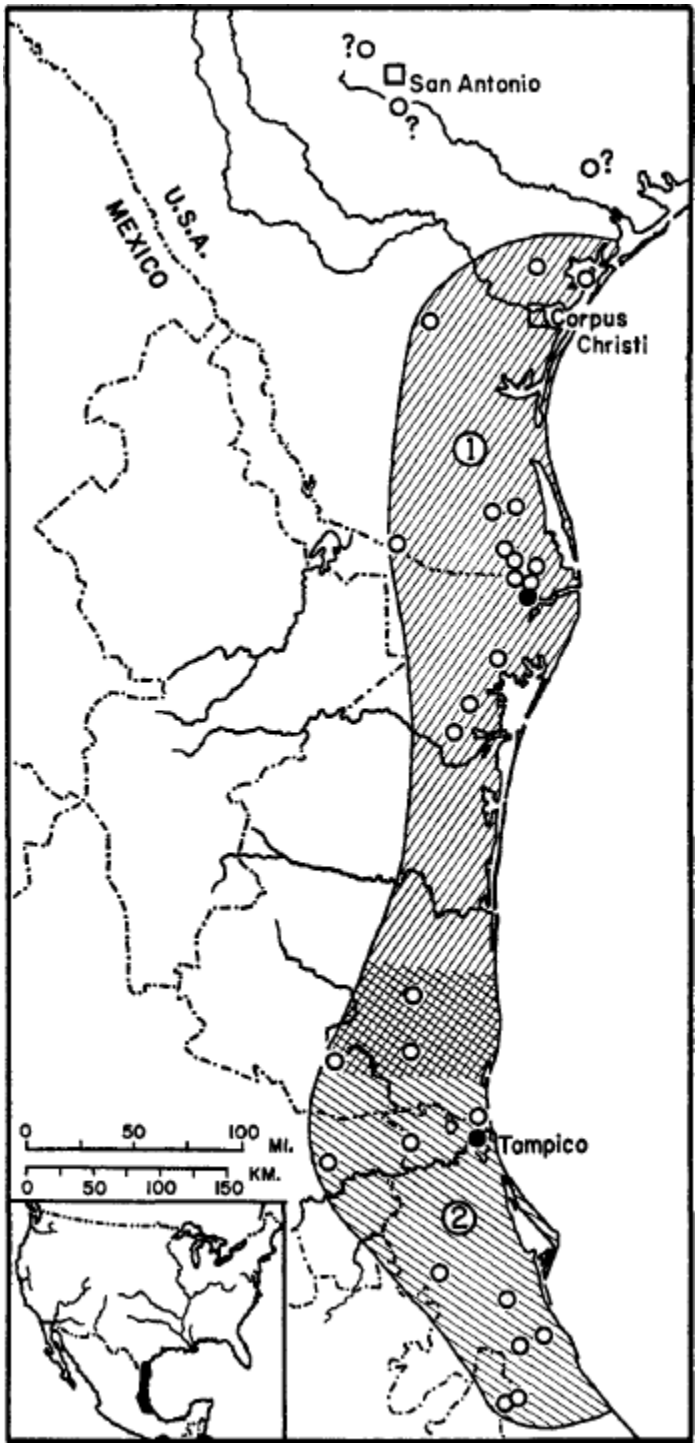

Figure 2. Published range distribution by Mecham (1968b). Solid symbols indicate type localities with hollow symbols marking other known locations. Overlap of shaded region indicates zone of intergradation. Dubious records with "?" beside them.

increasing since 1940 (Jahrsdoerfer & Leslie, 1988; US Census Bureau, 2010): Cameron County experienced a >400% increase while Hidalgo County showed a >800% increase. Such substantial growth has invariably lead to increased impact on local ecology. Human impacts on native LRGV vegetation have been drastic. In the past hundred years, 95% of native brushlands and 99% of riparian vegetation have been eliminated for urbanization, agriculture, and recreation (Jahrsdoerfer & Leslie, 1988).. Intensive agriculture and grazing is present throughout the LRGV (Moulton, Dahl, Petersburg, & Dall, 1997), and brings with it a variety of non-native and potentially invasive grasses (Rappole, Russell, Norwine, & Fulbright, 1986) while simultaneously allowing for encroachment of woody vegetation (Scott, 1996; Rappole, Russell, Norwine, & Fulbright, 1986). Desertification (Rappole et al., 1986) and fragmentation (Scott, 1996) have resulted from over-grazing and road construction, leading to fears of substantial habitat loss (Judd, 1985; Rappole & Klicka, 1991).

Irrigation practices in the LRGV are also having a significant impact on native wetlands. Prior to flood control projects in the early 1900's, the Rio Grande flooded the LRGV 23 times between 1900 and 1939 (Ramirez, 1986). Additional post-WWII projects, such as dams and irrigation canals, have impacted historical water regimes within the valley to where annual flood cycles that would have filled ponds and resacas (Chambers, 1930) have been stopped (Jahrsdoerfer & Leslie, 1988; Judd, 1985). As a result, local wetlands with historical BSN sightings in Texas have disappeared or have been drained for crop farming (Irwin, 1993; Judd, 1985), and potential palustrine habitats across the valley have been in steady decline (Moulton et al., 1997).

Black-spotted newts in the LRGV have been documented in a variety of habitats including Sabal palm forests, thorn scrub marshlands, and agricultural fields (Judd, 1985; Mecham, 1968a; Rappole & Klicka, 1991; Thornton, 1977). To date, there has not been a genetics study focused on

this species, a major concern considering the suspected population declines across its range (Flores-Villela et al., 2008). Until now, the best range estimate was provided by Mecham (1968) with identification books providing minor changes. Additionally, the potential scarcity and enigmatic nature of the species makes searches difficult, even in known localities. Without a more modern depiction of the species range in congruence with ecological variables, future studies will continue to be hampered by vague location descriptions and a generalized range. This study is divided into three sections (1) a range distribution model developed from occurrence records and associated environmental characteristics, (2) surveying technique assessment including novel strategies, and (3) a genetic analysis of the recognized subspecies and preliminary analysis of populations within the LRGV.

CHAPTER II

MAXENT HABITAT MODELING: POPULATION DISTRIBUTION OF THE BLACK-SPOTTED NEWT

Introduction

Estimates of geographic distribution are invaluable references for conservation of rare and endangered species. Probabilistic species distribution models (SDMs) have been generated for a multitude of plant (Kumar & Stohlgren, 2009; Loiselle et al., 2008) and animal species (Andersen & Beauvais, 2013; Anderson & Raza, 2010; Groff, Marks, & Hayes, 2014; Hernandez, Graham, Master, & Albert, 2006; Pearson, Raxworthy, Nakamura, & Townsend Peterson, 2007; Ward, 2007), including salamanders (Milanovich, Peterman, Nibbelink, & Maerz, 2010b; Wooten, Camp, & Rissler, 2010), demonstrating a wide breadth of applicability and effectiveness (Ha & Shilling, 2017; Phillips & Dudík, 2008). Species distribution models are commonly generated using climate (Andersen & Beauvais, 2013; Groff et al., 2014; Jackson & Robertson, 2010; Phillips & Dudík, 2008) and soil variables (Frisbie & Wyman, 1991; Wyman, 1988), georeferenced with species occurrence data within geographic information systems (GIS) databases and can also be used for modeling climate change impacts (Hijmans & Graham, 2006; Milanovich et al., 2010b). Uses for SDMs are vast with potential for modeling cryptic (Lozier, Aniello, & Hickerson, 2009; Pearson et al., 2007; Rissler, Apodaca, & Weins, 2007), invasive (Feldmeier et al., 2016; Rodrigues, Coelho, & Diniz-Filho, 2016; Stohlgren, Jarnevich, Esaias, & Morisette, 2011; Ward,

2007), or endangered (Kumar & Stohlgren, 2009; Wilting et al., 2010; York et al., 2011) species. Once generated, these predictive models can be overlaid on to regional maps to focus investigations.

Recently, many SDMs have been generated using the Maximum Entropy (MaxEnt) algorithm. This algorithm has been used successfully to predict range distributions of geckos (Pearson et al., 2007), moles (Jackson & Robertson, 2011), birds (York et al., 2011), amphibians (Giovannini, Seglie, & Giacoma, 2014; Groff et al., 2014; Milanovich, Peterman, Nibbelink, & Maerz, 2010a), and fungal diseases (Feldmeier et al., 2016) even with minimal occurrence data. MaxEnt models are derived by using available environmental data while minimizing constraints or maximizing model entropy (Phillips, Anderson, & Schapire, 2006). Research has suggested that MaxEnt models are more accurate in their predictions than other available algorithms (Elith et al., 2006; Phillips et al., 2006; Ward, 2007), showing a greater consistency in areas of higher probability and lower population density in low probability areas (van Proosdij, Sosef, Wieringa, & Raes, 2016). Furthermore, simulation studies on SDMs have found that few records may be required for moderate to high model accuracy (van Proosdij et al., 2016), with MaxEnt showing the greatest predictive power across sample sizes compared to other commonly used modeling algorithms such as BIOCLIM, generalized linear modeling, or DOMAIN (Pearson et al., 2007; Wisz et al., 2008).

The Black-spotted Newt, *Notopthalmus meridionalis* (BSN), is separated into northern (*N. m. meridionalis*, Texas BSN) and southern (*N. m. kallerti*, Mexican BSN) subspecies (Mecham, 1968b) with a poorly documented intergrade area in southern Tamaulipas, Mexico. International groups have classified the Black-spotted Newt as globally endangered (International Union for Conservation of Nature) and critically imperiled (NatureServe), while the species is federally

protected in Mexico (Secretaría de Medio Ambiente y Recursos Naturales, 2010), and state listed in Texas (Texas Parks and Wildlife Department, 2000). Because of their cryptic nature and the ephemeral nature of their aquatic habitats, our understanding of their habitat requirements and distribution is limited, hampering future conservation efforts. Amphibians are widely regarded as indicator species (Evans et al., 2016; Waddle, 2006; Wilson & McCranie, 2003), but with habitat destruction and fragmentation assumed as major factors causing population declines (Flores-Villa et al., 2008) in both BSN subspecies we may be losing a primary indicator of environmental quality. Development of SDMs would greatly inform future investigations for both subspecies, and greatly inform conservation practices.

Mecham (1968a) has the most widely accepted distribution model for *N. meridionalis*. Over the past 50 years, multiple sightings have been made beyond this scope while historic locations have failed to produce any records within that time. The aim of this research was to develop SDMs for each BSN subspecies (*N. m. meridionalis* and *N. m. kallerti*) using MaxEnt algorithms with comparative analysis of climate and soil parameters between subspecies. This study had three goals: (1) generate predictive models for BSN subspecies distributions, (2) identify environmental factors that could distinguish subspecies habitats, and (3) assess habitat overlap between BSN subspecies.

Methods

Records

Records were collected from four sources: scientific literature, online databases (https://www.gbif.org/, http://vertnet.org/, https://www.inaturalist.org/, https://www.idigbio.org/, and http://arctos.database.museum/SpecimenSearch.cfm), Texas Natural Diversity Database (TXNDD), and recordings from this study. When available, records were cross-referenced

between sources and amended based on all available data. Historic data was combined with data from this study to develop a master list of all known documented BSN sightings including date, location, collector, and museum catalog number if available. Two records were excluded due to subspecies uncertainty (Mecham, 1968ab) within the intergrade zone. In addition, Texas records for the Central Newt (*N. v. louisianensis*) were collected through online databases to provide a predictive range of a well-studied congeneric species for comparison. Repetitive samples collected from the same site on the same day were represented as a single record to avoid sampling bias; life stage collections were distinguished in recording but treated as one data point for modeling purposes. Records without GPS coordinates were estimated based on location description, if possible, to the nearest 5 km. If no GPS or location descriptions were provided, the record was not used for modeling. All coordinates were converted to the WGS 1984 coordinate system.

Site recordings from this study were used for modeling with additional records given priority assessments based on criteria used by Andersen & Beauvais (2013). From historical records, sites were given priority designations based on geographical specificity and cross-validation between studies, with records of high priority used as samples for analysis. Sites with specific geographic coordinates were given highest priority and thus used as sample records; when coordinates were unavailable, priority level was based on detail of location description (example: "Hwy 101, 35.1 mi N Jct. Hwy 97 in pond 70 m x 15 m x 1 m" given higher priority than "El Tejon, 9.5 mi N"). Records with a >5 km uncertainty radius were removed from consideration. From final list, records were removed based on above criteria until no records were within 5 km of each other to reduce sampling bias.

MaxEnt

Environmental layers were of four categories: climate, elevation, soil chemistry, and soil particle size. Nineteen climate variables (Table 1) at 1-km resolution were collected from WorldClim version 2 database (Fick and Hijmans 2017). Elevation data at 30-m resolution was provided by USGS Earth Explorer (USGS, NGIA, and NASA 2000). Soil layers at 250-m resolution were provided by SoilGrids250m (Hengl et al., 2017). Soil data was collected to represent three levels of soil: surface, 30-cm depth, and 1-m depth. Soil chemistry data was collected on cation exchange capacity, organic carbon content, KCl pH, and H_2O pH. Soil particle layers were collected based on particle size, representing percentages of coarse material, sand, silt, and clay. Bulk density layers for soil were also included. Final raster layers were limited to the following extent: 32 N, 94 W, 17 N, 101 W. All layers were resampled to a 30 m x 30 m scale to match elevation data resolution, providing a 30-m minimum resolution.

Table 1. List of climate variables associated with WorldClim database.

Code	Variable
BIO01	*Annual Mean Temperature*
BIO02	*Mean Diurnal Range (Mean of Monthly Temperature Range)*
BIO03	*Isothermality (BIO2/BIO7) (* 100)*
BIO04	*Temperature Seasonality (Standard Deviation *100)*
BIO05	*Max Temperature of Warmest Month*
BIO06	*Min Temperature of Coldest Month*
BIO07	*Temperature Annual Range (BIO5-BIO6)*
BIO08	*Mean Temperature of Wettest Quarter*
BIO09	*Mean Temperature of Driest Quarter*
BIO10	*Mean Temperature of Warmest Quarter*
BIO11	*Mean Temperature of Coldest Quarter*
BIO12	*Annual Precipitation*
BIO13	*Precipitation of Wettest Month*
BIO14	*Precipitation of Driest Month*
BIO15	*Precipitation Seasonality (Coefficient of Variation)*
BIO16	*Precipitation of Wettest Quarter*
BIO17	*Precipitation of Driest Quarter*
BIO18	*Precipitation of Warmest Quarter*
BIO19	*Precipitation of Coldest Quarter*

Two models for each focal subspecies were created: a constrictive model with all variables and a generalized model. Generalized models were based on an iterative process described by Andersen and Beauvais (2013) wherein correlates were parsed from further analysis to reduce bias based on permutation importance estimated from MaxEnt modeling. Three iterations of this procedure were done with variables contributing less than 1% average importance removed for the next iteration (Evangelista, Stohlgren, Morisette, & Kumar, 2009; York et al., 2011). Pearson correlation analysis was conducted across entire examined extent for each environmental data category separately (climate, soil chemistry, soil particle size) with a single representative with the highest average percent contribution across all models chosen amongst correlates with coefficient >|0.8| (Andersen & Beauvais, 2013; Lozier et al., 2009; York et al., 2011). Modeling was performed with MaxEnt version 3.4.1 (Phillips, Dudik, and Schapire, 2017) and GIS modeling was performed using ArcGIS 10.4.

Analysis

Raster data for each variable was extracted to each species point. An additional four data points (two *N. m. meridionalis* and two *N. v. louisianensis*) were removed due to lack of variable data –a result of being in water bodies where soil data could not be gathered. Cook's distances were calculated using the cooks.distance function in R, with samples greater than four times the mean identified as outliers. Permutational analysis of variance (PERMANOVA) and BioEnv analyses were conducted using the adonis and bioenv functions from the VEGAN R package (Dixon, 2003) respectively to distinguish variables between subspecies. Environmental dissimilarity matrices for PERMANOVA were derived using Gower's algorithm to account for non-Euclidean geometry in variable data. BioEnv analysis was conducted using Pearson correlation and Gower metrics. Multidimensional scaling (MDS) was conducted on subspecies

point data using the pcoa function from the APE package for R (Paradis, Claude, & Strimmer, 2004) and plotted using the ggplot2 package (Wickham, 2016).

Variable response curves, jackknife responses, and variable contributions were generated by MaxEnt to describe within model variability and identify influential variables for each subspecies (Groff et al., 2014; Pearson et al., 2007). Bootstrap analysis and subsample tests were not conducted due to minimal data, following Andersen and Beauvais (2013). All raster and point data analyses were conducted using R-3.3.3 (R Core Team, 2013).

Probability values between BSN subspecies were compared based on constrictive and generalized models using paired t-tests and visualized with boxplots based on values extracted within the intergrade zone across 1000 random points. Intergrade zone probabilities from constrictive and generalized models were assessed independently, with comparisons conducted between BSN subspecies.

Results

Pearson correlation analysis found the following sets (surface, mid, and bottom levels) had correlation above 0.8 cut-off: cation exchange capacity (>0.81), clay percentage (>0.89), coarse material percentage (>0.95), sand percentage (>0.94), and silt percentage (>0.94). All pH layers (H2O and KCl) were found to be correlated (>0.85). Organic carbon content and bulk density layers were both correlated at low- and mid- levels (0.905 and 0.975 respectively), but surface levels were not correlated. BioClim layers contained five sets of correlated variables, only Mean Temperature of Driest Quarter (BIO09) was uncorrelated to any other climate variable. Bulk density, percent sand, and percent silt variables were not found to be associated with any species in the constrictive model (where all variables were used) and were removed from further modeling. The final set of variables used for constrictive modeling can be found in Table 2.

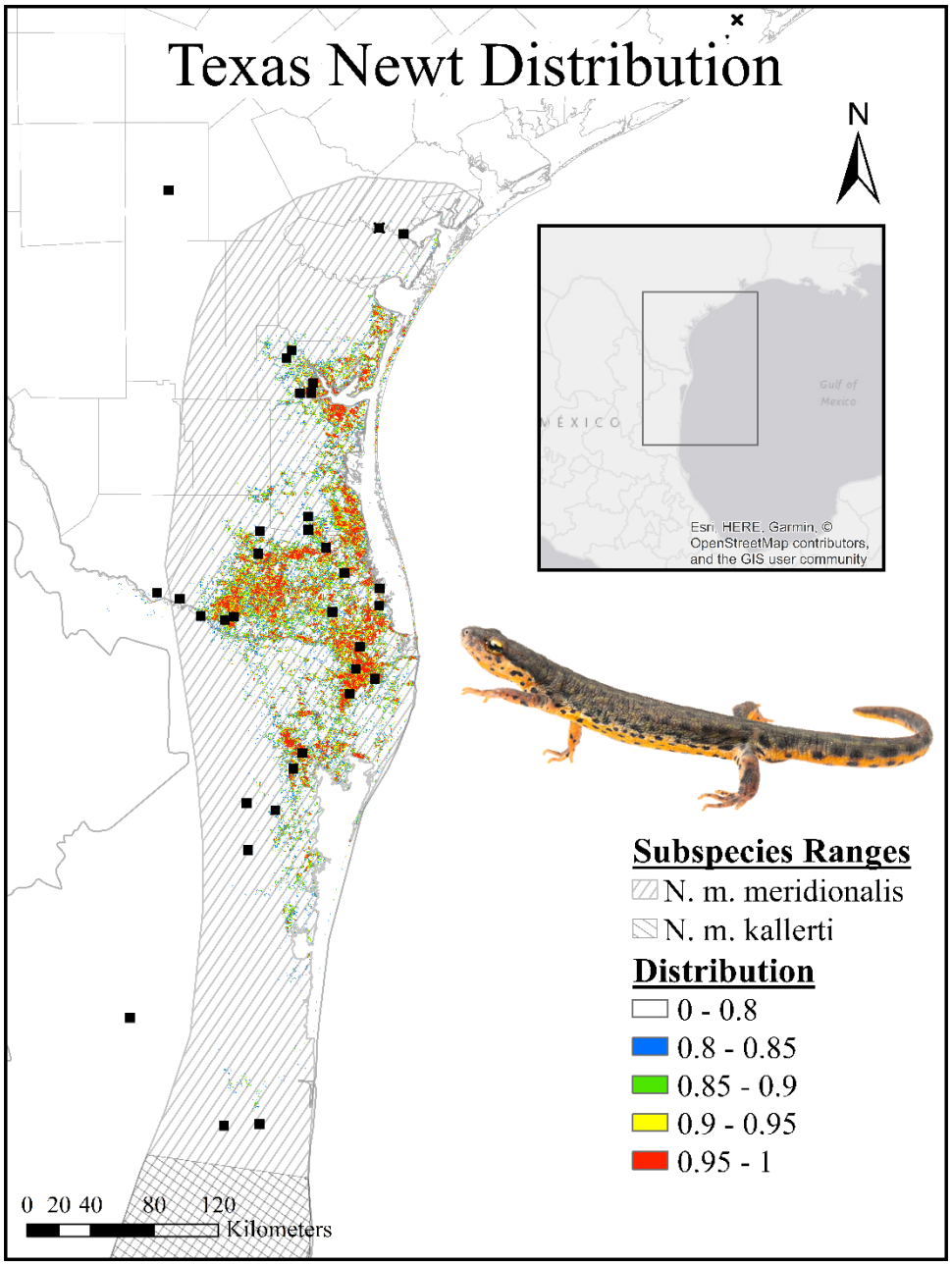

Figure 3. Constrictive model range distribution map for the Texas Black-spotted Newt (*N. m. meridionalis*) using all available variables. Five high probability regions appear across this model: SE Cameron County, SE Hidalgo County, coastal Willacy County, proximal Baffin Bay, and NE Tamaulipas. While sand was not found to be a significant factor, probability declines dramatically at interface of sand sheet across Kenedy and Kleberg counties. Percent probability >80% shown.

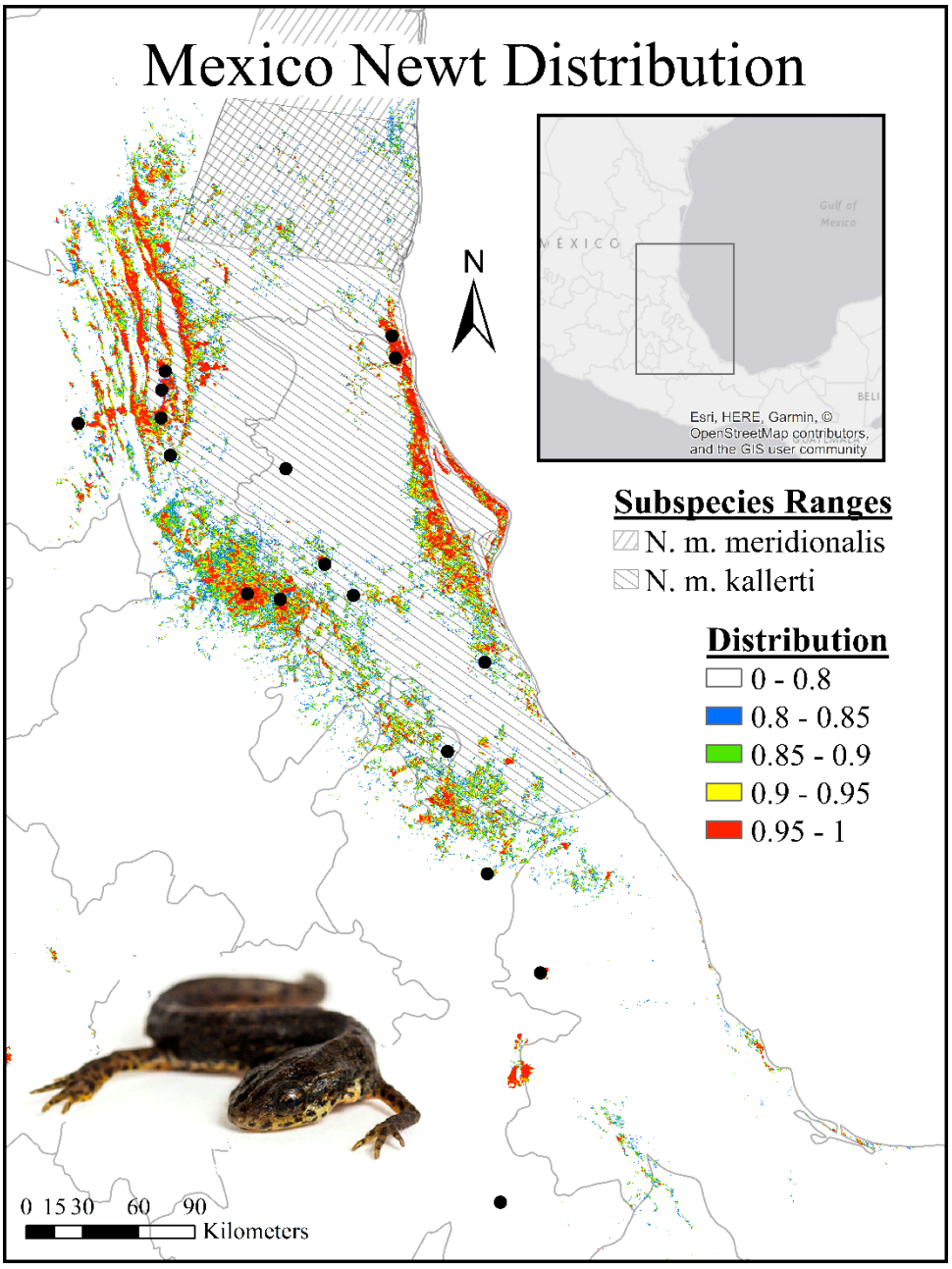

Figure 4. Generalized model range distribution map for the Mexican Black-spotted Newt (*N. m. meridionalis*) using variables defined in Table 2. Highest probabilities found along coastline and foothills. Low estimated connectivity across valley between high probability regions suggests long-term genetic separation may have occurred. Percent probability >80% shown.

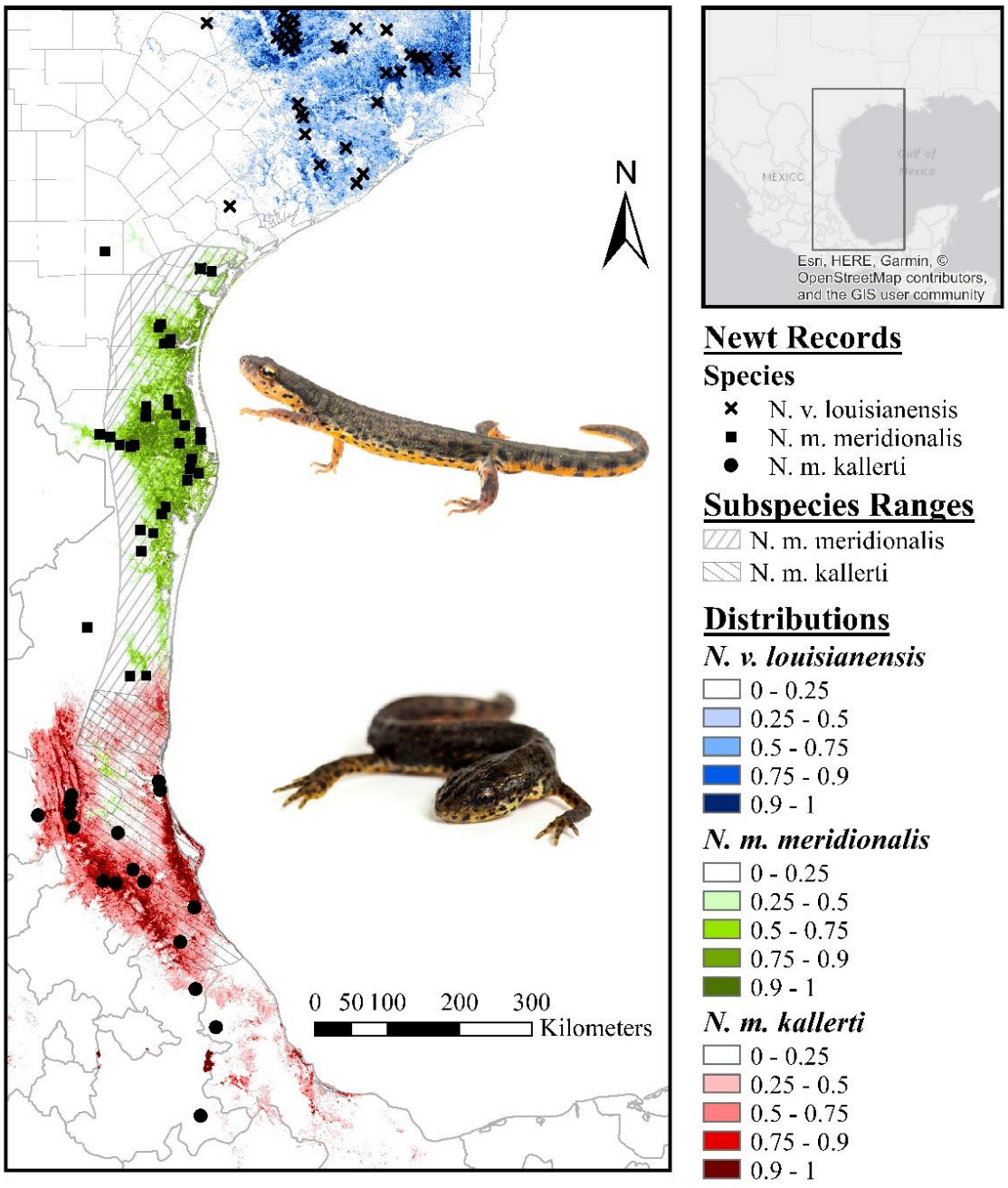

Figure 5. Constrictive model range distribution map for the Central Newt (*N. v. louisianensis*), Texas Black-spotted Newt (*N. m. meridionalis*), and Mexican Black-spotted Newt (*N. m. kallerti*). Compared to generalized models, *N. m. meridionalis* follows coastline and riverine waterways slightly more while *N. m. kallerti* shows significant reduction in northern and southern extents.

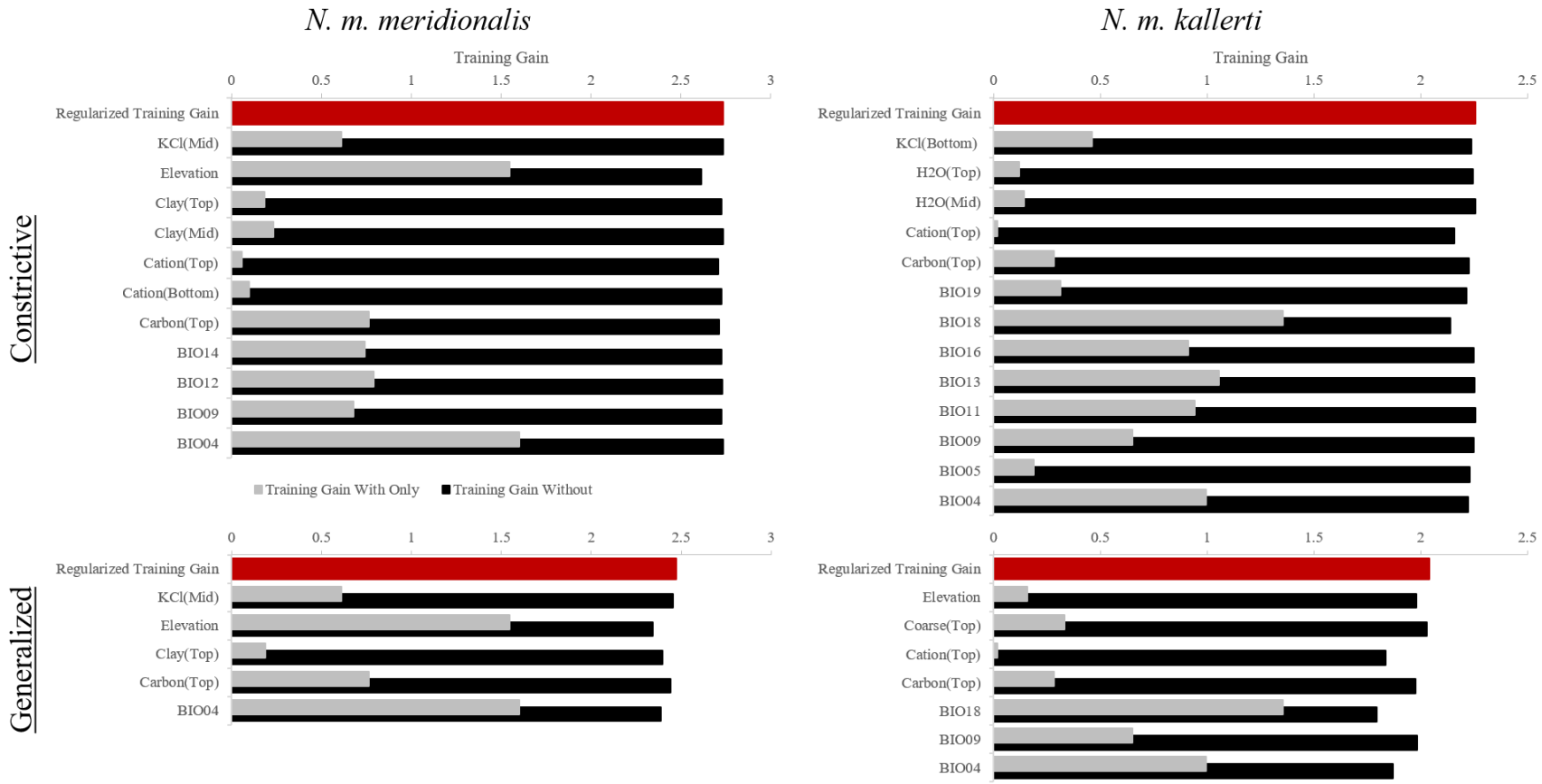

Figure 6. Jackknife results of constrictive and generalized models for *N. m. meridionalis* and *N. m. kallerti*. To emphasize variable impacts, constrictive model figures use variables with a >1% contribution or importance to the model, while generalized model figures use variables with a >5% contribution or importance to the final model. Maximal total gain a single variable can explain is represented in gray, while maximum gain explained without said variable is represented in black, compare to regularized training gain in red.

Table 2. Variables used for generalized modeling of *N. m. meridionalis* and *N. m. kallerti*

Code	Variable
BIO02	*Mean Diurnal Range*
BIO04	*Temperature Seasonality*
BIO09	*Mean Temperature of Driest Quarter*
BIO18	*Precipitation of Warmest Quarter*
Carbon (Top)	*Organic Carbon Content at Surface*
Cation (Top)	*Cation Exchange Capacity at Surface*
Clay (Top)	*Clay Percentage at Surface*
Coarse (Top)	*Coarse Material Percentage at Surface*
Elevation	*Elevation*
KCl pH (Mid)	*pH Measured by KCl at 30-m Depth*

In total, 32 Texas BSN records and 17 Mexican BSN records were used for generating MaxEnt models. Two Mexican BSN records were found to be outliers but were retained due to location precision with GPS coordinates. Multidimensional scaling of data points with 95% confidence interval (Figure 7) indicated minor habitat overlap between BSN subspecies and separation of both BSN subspecies from *N. v. louisianensisi*, similar to MaxEnt models (Figure 5). Axis 1 (PCO1) accounted for 50.05% of variation and axis 2 (PCO2) 21.08%. Between BSN subspecies two distinguishing variables were identified using PERMANOVA: Temperature Seasonality (standard deviation * 100) (BIO04; $R^2 = 0.839$; $p < 0.001$) and Precipitation of Warmest Quarter (BIO18; $R^2 = 0.065$; $p < 0.001$) (Figure 8). BioEnv analysis found the best set of environmental variables correlative to point data ($R = 0.836$) was a combination of Temperature Seasonality (BIO04) and Precipitation of Warmest Quarter (BIO18). Organic carbon content ($R^2 = 0.026$; $p < 0.001$) was significant but not well-correlated to regional distribution patterns.

Both Texas BSN models had high AUC values constrictive model – 0.981 and generalized – 0.974). In both models, elevation had the greatest contribution (constrictive - 57.6%; generalized – 60.8%) and importance (constrictive - 68.9%; generalized – 67.0%) (Table 3, Figure 3), Constrictive MaxEnt modeling for the Texas BSN revealed five major regions based on 80%

predictive probability: northcentral Tamaulipas, southern Cameron County into northern Tamaulipas, SE Hidalgo County, coastal Willacy and Kenedy counties, and the coastal grasslands around Baffin Bay (Figure 3). In the constrictive model, KCl (mid-range) pH and organic carbon content (at surface) were each found to have a significant contribution (>5%) to the final model (Table 3).

Table 3. Variable percent contribution or importance for each model (general and constrictive) of both Black-spotted Newt subspecies (*N. m. meridionalis* and *N. m. kallerti*). Tables represent only variables with a >1% model contribution or importance. Variables arranged by percent contribution.

Constrictive

N. m. meridionalis		
	Contribution	**Importance**
Elevation	57.5847	68.8719
KCl (Mid)	16.1942	0
Carbon (Top)	12.1875	2.6007
BIO14	2.6488	14.5528
Clay(Top)	1.8749	1.8106
BIO04	1.332	1.1124
Clay (Mid)	1.2495	0
Cation (Bottom)	0.4461	1.024
BIO09	0.4377	1.2119
Cation (Top)	0.3746	2.6563
BIO12	0.0754	1.0253

N. m. kallerti		
	Contribution	**Importance**
BIO18	64.1852	32.3099
H2O (Mid)	5.8488	0
BIO05	4.6986	1.622
BIO04	4.1793	26.7217
Cation (Top)	4.15	4.2464
H2O (Top)	3.9799	3.6544
BIO13	3.1657	1.1287
BIO11	2.8473	0
BIO19	2.8018	1.7167
BIO09	1.2017	15.3203
Carbon (Top)	0.9805	2.3679
KCl (Bottom)	0.6552	8.2324
BIO16	0.2342	1.9219

Generalized

N. m. meridionalis		
	Contribution	**Importance**
Elevation	60.7517	66.9503
KCl (Mid)	17.0526	1.1048
Carbon (Top)	13.9205	3.2686
BIO04	4.4737	25.7261
Clay (Top)	3.2121	2.02

N. m. kallerti		
	Contribution	**Importance**
BIO18	67.6018	57.8302
BIO04	16.0416	6.497
Cation (Top)	5.5854	15.3093
BIO09	4.5136	13.3606
Elevation	3.5473	2.101
Carbon (Top)	1.5373	2.6855
Coarse (Top)	1.1053	2.1546

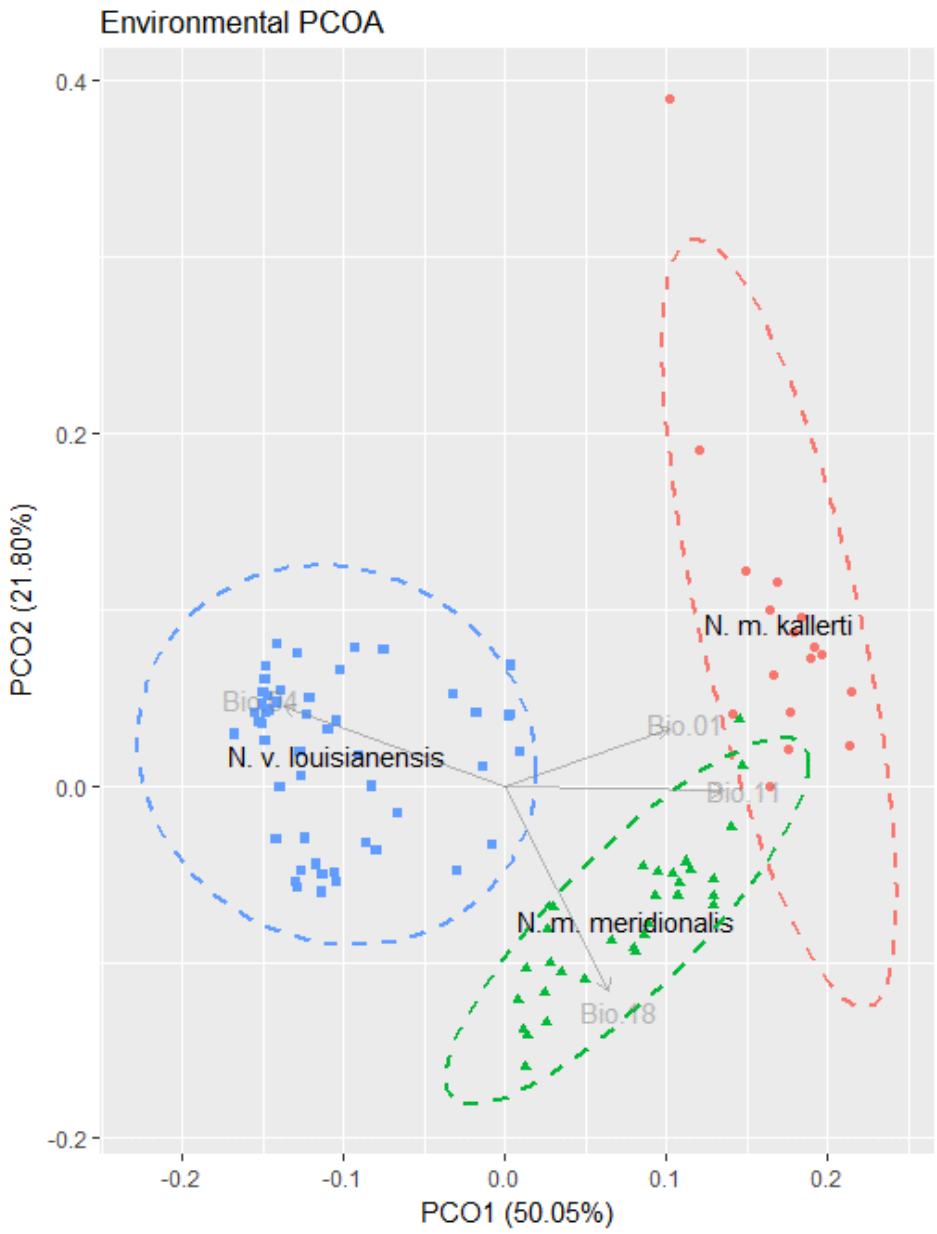

Figure 7. PCoA (MDS) plot for the Central Newt (*N. v. louisianensis*, blue), Texas Black-spotted Newt (*N. m. meridionalis*, green), and Mexican Black-spotted Newt (*N. m. kallerti*, red), showing environmental divergence between three newt subspecies. Vectors displayed representing directional influence of top four influential PERMANOVA identified variables: BIO1 (Mean annual temperature), BIO4 (Temperature seasonality), BIO11 (Mean temperature of coldest quarter), and BIO18 (Precipitation of warmest quarter).

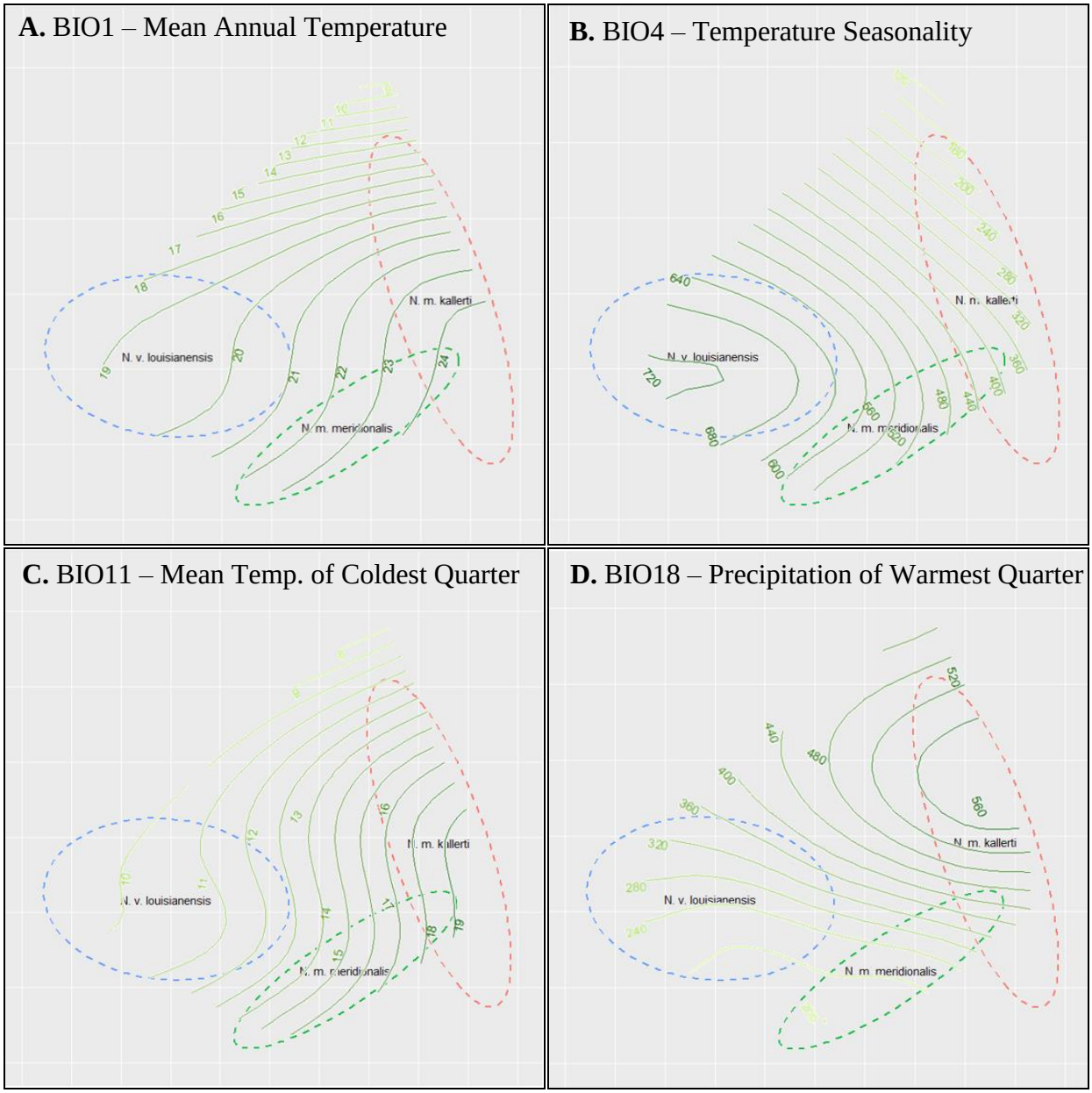

Figure 8. PCoA (MDS) plots for the Central Newt (*N. v. louisianensis*, blue), Texas Black-spotted Newt (*N. m. meridionalis*, green), and Mexican Black-spotted Newt (*N. m. kallerti*, red), with variable contour lines depicting values across MDS ordination. (B) BIO4 (Temperature seasonality) and (D) BIO18 (Precipitation of warmest quarter) were both found to be significantly different between Black-spotted Newt subspecies, while (A) BIO1 (Mean annual temperature) and (C) BIO11 (Mean temperature of coldest quarter) had significant contribution between *N. v. louisianensis* and *N. meridionalis.*

Mexican BSN models also had high AUC values (constrictive model - 0.977 and generalized model – 0.971). Precipitation of Warmest Quarter (BIO18) had the greatest contribution and highest importance in both models (constrictive: 64.2% and 32.3%, respectively; general: 67.6% and 57.8%, respectively; Table 3 and Figure 6). Additionally, Temperature Seasonality (BIO04) was within the top 5 variables in contribution and importance for both models. MaxEnt models for Mexican BSN partition the subspecies into two larger regions along the coastline and in the foothills based on an 80% probability cut-off (Figure 4).

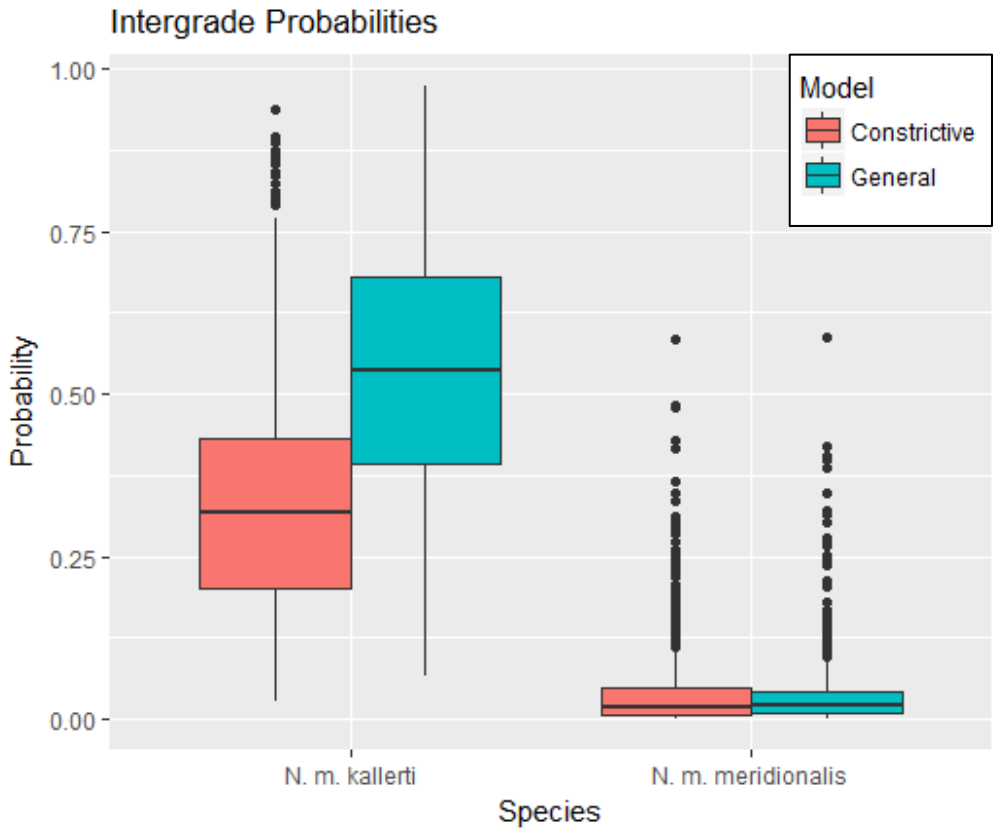

Figure 9. Distribution probability measurements for the Black-spotted Newt subspecies (*N. m. meridionalis* and *N. m. kallerti*) within intergrade zone based on constrictive (red) and general (blue) models.

Mexican BSN models were consistently impacted by more climate variables than Texas BSN (Table 3, Figure 4) across both constrictive and generalized models. For each constrictive model a single soil chemistry variable differed between subspecies (Texas BSN – KCl (mid);

Mexican BSN – Cation (surface)). Similarly, each constrictive model had a single soil material variable which differed by subspecies, but neither had an importance or contribution >5% for their respective models.

Out of 1,000 random points assigned for intergrade zone analysis, 12 were removed due to N/A values. Paired t-test results for both models found Mexican BSN to be more probable in the intergrade zone than Texas BSN (General: $t = -73.637$, $df = 988$, $p < 0.001$; Conservative: $t = -51.045$, $df = 988$, $p < 0.001$; Figure 9) suggesting habitat in that area favors the Mexican BSN.

Discussion

With relatively few records used for model generation, predictions should be recognized as determinant of regional likelihood rather than absolute species limits (Giovannini et al., 2014; Hernandez et al., 2006; Pearson et al., 2007; van Proosdij et al., 2016). For Texas BSN studies, the constrictive model is recommended (Figure 3) as it provides the clearest regional differentiation and is based on a greater number of records (Wisz et al., 2008). Comparatively, the generalized model is suggested for Mexican BSN in order to not discount a more expansive range (Wisz et al., 2008). While smaller sample sizes may cause over-prediction by modeling algorithms (Jiménez-Valverde, Lobo, & Hortal, 2008; Sinclair, White, & Newell, 2010), it is also likely that the true extent of a cryptic species' range is not fully accounted for by the available records at small sample sizes (Pearson et al., 2007) Future investigations should focus on regions identified above, especially where historical records are available as our investigations primarily involved Cameron County with few successful locations in Hidalgo and Willacy counties. Despite minimal to no data in some of these regions, MaxEnt models still predicted occurrences in regions with historical records providing confidence in these models.

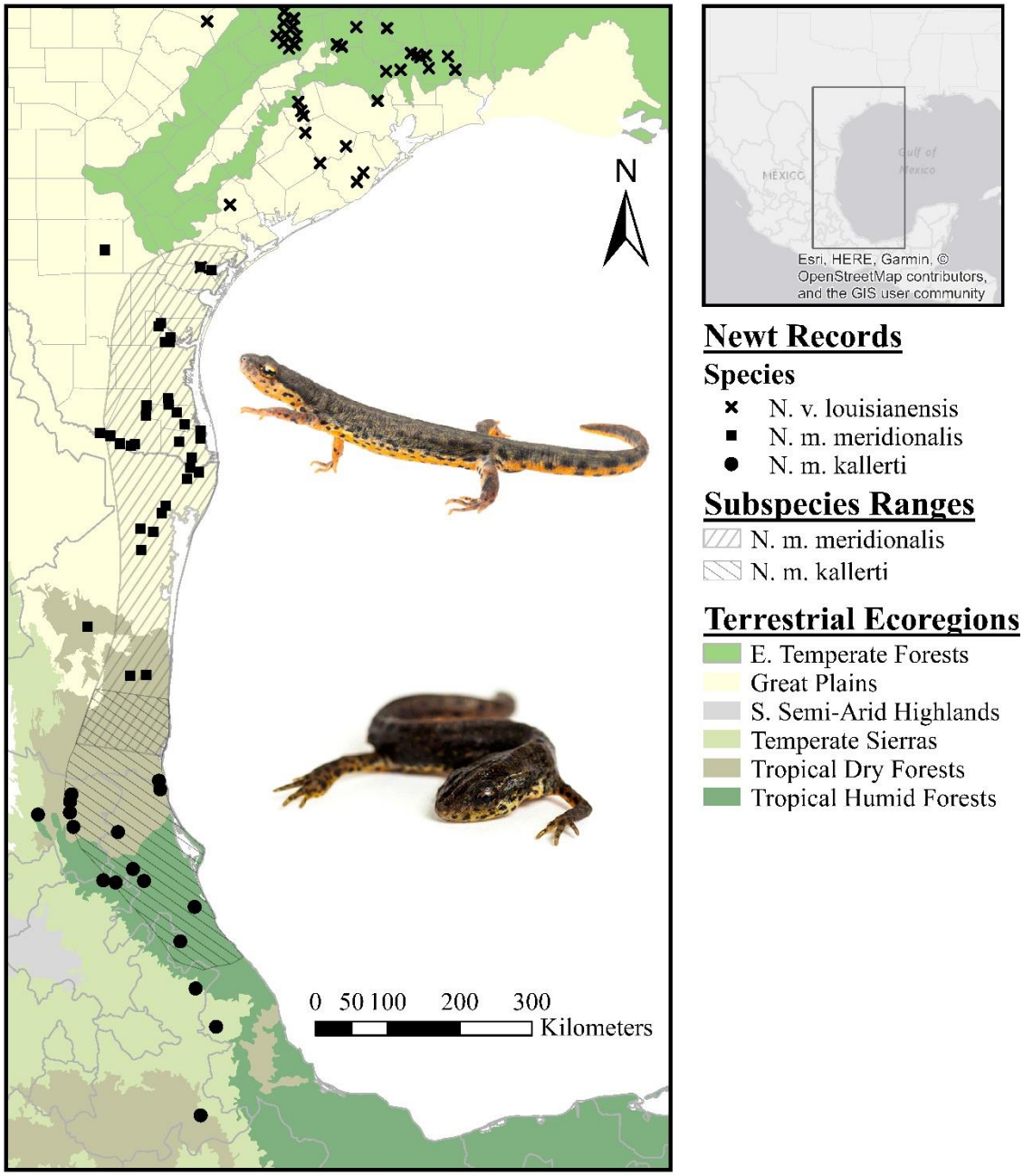

Figure 10. Terrestrial ecoregion (Level I) map displaying used records for the Central Newt (*N. v. louisianensis*), Texas Black-spotted Newt (*N. m. meridionalis*), and Mexican Black-spotted Newt *N. m. kallerti*), highlighting regional separation between Black-spotted Newt subspecies. Tropical Forest ecoregions follow *N. m. kallerti* distribution, while *N. m. meridionalis* is almost exclusive to Great Plains ecoregions. MaxEnt probabilities for *N. m. kallerti* similarly follow Tropical Forest ecoregion boundaries (see Figure 5).

Additionally, both Texas BSN models provide support for soil variables being influential in distribution. Soil qualities have been found to be informative for other species (Groff et al., 2014; Jackson & Robertson, 2011; Renan et al., 2017), and BSN individuals have beenfound in cavities (Bare & Kline, 2017; Rappole & Klicka, 1991) suggesting a potential influence of soil qualities. Future studies should investigate soil measures (pH, salinity, moisture content, water retention) at known localities to determine potential hospitable ranges for this species.

Based on environmental variable associations from MaxEnt models, the intergradation zone has a greater propensity towards Mexican BSN. However, within the intergradation zone newts are described as having morphological characters more closely related to those of the Texas BSN (Mecham, 1968b) based on 40 specimens across two sites (16 from Gonzales, Tamaulipas and, 24 from Sierra de Tamaulipas). This divergence between environment and morphology provides further support for the subspecies delineation. In salamander species color variation (see *Ensatina eschscholtzii* in (Stebbins, 2003)), head morphology (Van Buskirk & Schmidt, 2000; Walls, Belanger, & Blaustein, 1993), and body length (Brandon, 1965) are relatively malleable, even within *Notophthalmus* (Mecham 1967ab, 1968a). In addition, interbreeding among BSN subspecies has not been documented in the literature, despite potential for such examination in lab animals by McReynolds (1968) and Mecham (1968b). Future work should prioritize collection of DNA from potential intergrades as this species is federally protected in Mexico (Secretaría de Medio Ambiente y Recursos Naturales, 2010) and globally endangered (IUCN). Laboratory crossings may also be of value to determine hybridization between BSN subspecies, but also between the Texas BSN and *N. v. louisianensis*.

Predictive models presented herein suggest greater scrutiny is needed regarding the current taxonomic grades of these subspecies. The “niche conservatism” hypothesis (NC), a broad

suggests sister taxa should exhibit similar niche requirements with overlapping range predictions (Kozak & Wiens, 2006, 2010; Wiens, 2004). Notable divergence of environmental variables and predictive ranges exist between the two BSN subspecies, counter to NC. At present, predictive models suggest allopatry may exist between BSN subspecies with minimal support for range overlap. In addition, BSN subspecies show significantly different abiotic environmental factor requirements correlating with independently defined ecoregion level I boundaries (Commission for Environmental Cooperation, 1997; Figure 10). Further investigation may be pertinent into ecoregion levels II, III, and IV. Range partitioning seen in Mexico BSN models do imply NC (compare to Fig1B in (Kozak & Wiens, 2006)), and warrants greater study.

Models in the present study relied on relatively few data points due to historical records exhibiting highly variable precision. While minimal, SDMs using fewer records have found to have high accuracy (Giovannini et al., 2014; Pearson et al., 2007; van Proosdij et al., 2016; Wooten et al., 2010), providing confidence in the models generated. More precise location data and a greater number of data points will be increase accuracy of BSN habitat predictions and environmental limitations, reducing regions of 'overprediction' (Pearson et al., 2007).

In the present study, land use data was not incorporated, even though habitat destruction is seen as a primary cause for amphibian habitat loss (Brum et al., 2013; Cushman, 2006; Ficetola, Rondinini, Bonardi, Baisero, & Padoa-Schioppa, 2015; Scott, 1996) because these models are meant to predict habitat based on environmental data and data used comes from multiple decades during which habitat destruction has occurred across the BSN range (Jahrsdoerfer & Leslie, 1988). Models presented here are an initial step to provide evidence of environmental requirements and guide future efforts.

CHAPTER III

HUNTING CRYPTIC AMPHIBIANS: NOVEL SURVEYING TECHNIQUES FOR THE BLACK-SPOTTED NEWT

Introduction

Since first described by Cope (1880), the Black-spotted Newt (*Notophthalmus meridionalis,* BSN) is an understudied, cryptic salamander species (Rappole and Klicka 1991; Judd 1985) that is threatened in Texas (Texas Parks and Wildlife Department, 2000) and endangered in Mexico (SEMARNAT 2002). Recorded BSN observations have mostly been from dip-netting surveys in breeding ponds (Judd 1985; Strecker 1922; Kazmaier 2009; Mecham 1968b; Rappole and Klicka 1991; Martin, Robins, and Heed 1954) or by debris searches (Kazmaier 2009; Mecham 1968a; Rappole and Klicka 1991; Thornton 1977), but newts can also be found on the surface during heavy rains (Thornton 1977; Taggart 1997) or underground (Rappole and Klicka 1991; Bare and Kline 2017). Compared to terrestrial salamander species (Gabriel F. Strain and Raesly 2006; G. F. Strain, Raesly, and Hilderbrand 2009; Regosin et al. 2005; Hicks and Pearson 2003; Bailey, Simons, and Pollock 2004), BSN detection can be unpredictable (Rappole and Klicka 1991; Judd 1985). Moreover, traditional sampling techniques can be expensive and time consuming (Davy, Kidd, and Wilson 2015; Katano et al. 2017). As such, there is a need for more sensitive BSN monitoring techniques.

Environmental DNA (eDNA) is a non-invasive technique for detecting water-borne species without direct capture. This novel technique detects species presence through genetic means

without an identifiable source. Most eDNA studies have used water samples (Ficetola et al. 2008; Bista et al. 2017; Thomsen et al. 2016; Dunker et al. 2016), but saliva (Wheat et al. 2016), feces (MacDonald and Sarre 2017), and soil samples (Turner, Uy, and Everhart 2015; Guardiola et al. 2016) have also been used. Additionally, eDNA is commonly used to target specific species (Pilliod et al. 2014; Biggs et al. 2015; Dunker et al. 2016; Takahara, Minamoto, and Doi 2013), communities (Bista et al. 2017; Shelton et al. 2016; Olds et al. 2016; Goldberg et al. 2011), or even specific individuals (Wheat et al. 2016). Additionally, eDNA protocols can be relatively inexpensive compared to traditional field studies (Klymus, Marshall, and Stepien 2017; Sigsgaard et al. 2015; Taberlet et al. 2012). This is especially useful when working with cryptic species or diffuse populations where extensive survey efforts may be required.

The first use of eDNA for macrofauna was developed to monitor invasive bullfrog (*R. catesbeiana*) populations in France (Ficetola et al., 2008). In the decade since, eDNA has been widely expanded upon and is frequently used for amphibian monitoring (Pilliod et al. 2013; Dejean et al. 2012; Goldberg et al. 2011; Ficetola et al. 2008; De Souza et al. 2016; Biggs et al. 2015; Valentini et al. 2016). Studies with fish (Takahara et al. 2012; Klymus et al. 2015; Nathan et al. 2014; Doi et al. 2015) and salamanders (Pilliod et al. 2013; Evans et al. 2016) have demonstrated a correlation between eDNA concentration and biomass, reflecting relative abundance values. As a result of both high sensitivity and degradation rates, localized activity levels can even be perceived through eDNA assays (De Souza et al. 2016). The ease of eDNA sampling has even made it available for use by citizen scientists (Biggs et al. 2015). With wide application and high sensitivity, eDNA offers a powerful tool for BSN studies.

Terrestrial surveys for this salamander species have proven difficult due to their cryptic nature and habit of seeking refuge in burrows and fissures (Bare and Kline 2017; Rappole and

Klicka 1991). Among local amphibian enthusiasts, the BSN is considered difficult to find, even considered a near impossibility unless heavy rains fill breeding ponds. While terrestrial activity has been documented (Taggart 1997; Judd 1985; Rappole and Klicka 1991; Thornton 1977), observations have been infrequent and generally have provided few accounts. Rappole and Klicka (1991) were successful in locating newts by digging in dried pond beds and wetlands. However, this runs the risk of severe damage to the animals and may be frowned upon by private landowners and refuge managers. It is apparent that traditional terrestrial techniques used for other amphibian species are only partially effective at detecting newts due to their fossorial behaviors.

Given the challenges listed above, the aim of this research was to develop additional tools to identify newts in both terrestrial and aquatic habitats. Both habitats are difficult to survey effectively to assess BSN presence and population size. The research objectives for this study were to: 1) Develop an eDNA protocol to detect BSN habitation in the often turbid and eutrophic waters of south Texas 2) Develop effective terrestrial cavity survey techniques and compare these against standard survey techniques.

Methods

Environmental DNA

Primer Validation with BSN Tissue DNA. Two primer sets were developed (BSN5 and BSN7) based on a published mitochondrial genome sequence for *N. m. meridionalis* (GenBank #EU880322), targeting conserved regions within Cytochrome Oxidase III (CoxIII) and NADH Dehydrogenase 2 (ND2) (Table 4). A nested primer set (BSN5.1) was designed to increase specificity and sensitivity of the eDNA assay and to aid in visualization of positive samples (Clusa et al. 2017; Ward et al. 2016) (Table 4).

Table 4. Primer sets successfully used for BSN detection in eDNA assays. Primer NewtFW5.1b was designed to better match the melting temperature of primer NewtRV5.1 for improved PCR product for nested set.

Primer Set	Primer	Sequence	Amplicon Length
eDNA 5	NewtFW5	AAAACCCCTCGTGCTTCCACT	276
	NewtRV5	TTGTTGCGGATTCTGTTGCTCG	
eDNA 5.1	NewtFW5.1a	CCTCTCTTGCTAATGAACCCTTATGC	182
	NewtFW5.1b	CTCTCTTGCTAATGAACCCTTATGC	181
	NewtRV5.1	GTTGCGGATTCTGTTGCTCGG	
eDNA 7	NewtFW7	ACCCCATTAGACCCATTTGAAGTT	171
	NewtRV7	GGCTTGGAGGGCGGTAAA	

Tissue DNA from a newt was extracted from a previously collected tail clip that had been stored in 75% ethanol at -20^{o}C. Collection followed Institutional Animal Care and Use Committee (IACUC) approved protocols (2013-005-IACUC Kline). Tissue DNA extraction was performed using the GenCatch Blood & Tissue Mini-Prep Kit (#1460050, Epoch Life Science, Sugar Land, TX, USA) following manufacturer's instructions for mouse tail DNA extraction. Polymerase chain reaction (PCR) was conducted in 25 µL reactions using tissue DNA (30 ng) with GoTaq HotStart Master Mix (Promega Corp, Madison, WI) for validation. Each reaction was run as follows: 2 min initial denaturation at 95^{o}C, followed by 35 cycles of 30 s 95^{o}C denaturation, 30 s 58^{o}C annealing, and 60 s 72^{o}C extension steps. Twenty microliters of each PCR product were run for 50 min in 1.5% agarose gel electrophoresis and visually examined for a band of expected size with a DNA ladder under UV transillumination. The resulting bands were extracted using a PCR cleanup kit (GenCatch PCR Cleanup Kit #2360050, Epoch Life Science, Sugar Land, TX, USA). Extracted DNA was quantified using a Nanodrop (ND 2000, Fisher Scientific, USA) and Sanger sequenced (Eurofins) to verify the PCR products by NCBI Blast search.

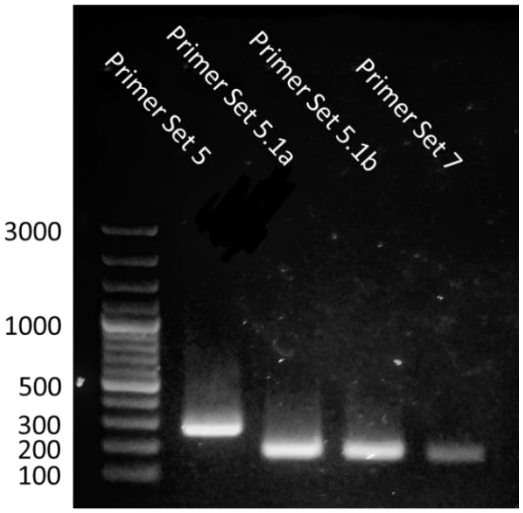

Figure 11. Amplification of BSN DNA from a tissue sample with eDNA primers 5, 5.1a, 5.1b, and 7 visualized by electrophoresis of agarose gel with expected band sizes for all primer sets (Set 5 – 276 bp, Set 5.1a – 182 bp, Set 5.1b – 181 bp, and Set 7 – 171 bp).

eDNA Protocol Validation. Water was collected from the BSN aquarium at Gladys Porter Zoo and diluted with reverse osmosis water (clear water) at 1:1000 dilution. The solution was mixed and allowed to sit for 30 min prior to vacuum filtration through Millipore brand (Bedford, MA, USA) polycarbonate track etch (PCTE) 47 mm diameter membrane filters with pore sizes 1.2 µm (#RTTP04700), 3.0 µm (#TSTP04700), 5.0 µm (#TMTP04700), and 8.0 µm (#TETP04700). Filters were folded twice and placed in 2 ml centrifuge tubes, with 700 µl of DNAzol storage solution (Molecular Research Center Inc., #DN-127, Cincinnati, Ohio).

Filters were stored at room temperature in DNAzol for up to 3 d prior to extraction. On day of extraction, all sample tubes were placed in a 55^{o}C heat bath for 30 min. PCTE filters were left in tubes because they would completely dissolve in chloroform. After heating, 500 µl chloroform #C298-500, ThermoFisher Scientific) was added to the DNAzol + PCTE filter trials. Solutions were then vortexed for 30 s and centrifuged for 30 s at 5000 rpm. The supernatant was extracted and placed in a new 2 ml centrifuge tube. DNA from

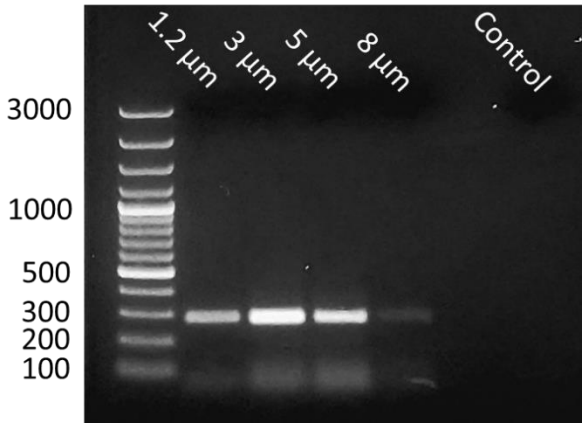

Figure 12. Bands of expected size (276 bp) were produced by primer set 5 through PCR amplification from filtrations across multiple filter sizes with dilutions of clear water. Filters were run with 250 mL of a 1:1000 diluted sample of aquarium water that contained black-spotted newts.

the DNAzol supernatant was precipitated by adding 500 µl absolute ETOH. Samples were centrifuged for 10 min at 13,000 rpm to pellet the DNA. The supernatant was discarded and the pellet was washed 2X by adding 500 µl 75% ETOH and vortexing for 30 s, followed by centrifugation at 7500 rpm. The DNA pellet was allowed to air dry for 30 min, then dissolved in 20 µl nuclease-free water. PCR was conducted in 25 µL reactions with GoTaq G2 HotStart Master Mix (M7422, Promega Corp, Madison, WI, USA), purified eDNA samples (< 30 ng), and 0.5 µL of each primer BSN5-FW and BSN5-RV (10 µmol). PCR conditions followed the procedure described above. Amplified products were visually compared for intensity and size of product that resulted.

Dilution with Pond Water. Following protocol validation, newt aquarium water was diluted 1:1000 with local pond water as a test of the eDNA sampling protocol in field conditions. Filtration was conducted using two methods: 1) Polycarbonate track etch (PCTE) membrane filters as describe above and 2) water manually filtered using coffee filters and a coffee press (# 80R08 AeroPress Inc., Palo Alto, CA, USA). Sample processing and DNA extraction proceeded as described in the validation above with the exception that coffee filters were manually removed using clean tweezers and compressed to extract as much DNAzol fluid as possible. All samples were quantified and run in PCR using the same conditions listed above. Volume of water filtered was recorded for a comparison of each filter type in field conditions. Amplified products were visually compared for intensity and size of product that resulted.

Results. Amplification of DNA from a BSN tissue sample with eDNA primers and visualized with agarose electrophoresis resulted in bands of expected sizes for primer sets BSN5, BSN 5.1a, BSN 5.1b and BSN7 (Figure 11). Sanger sequenced PCR products from each primer set all matched target regions of *N. meridionalis* (GenBank accession EU880322). Primer set BSN5 was

selected as the best primer set for use in eDNA trials because it had the greatest length and could also be used in nested PCR with primer set 5.1a or 5.1b for increased sensitivity.

Bands of expected sizes were produced by PCR amplification from 250 ml filtrations of clear water dilutions of aquarium extracts across all PCTE filter sizes (Figure 12). Intensity was highest in the 3 and 5 µm filtered extracts and lowest in the 8 µm sample with no visible band in the negative control (NTC) sample. Sequences from extracted bands matched target mitochondrial region for *N. meridionalis* (GenBank accession EU880322).

Filter Size	Volume
1.2 μm	100 mL
2.0 μm	50 mL
3.0 μm	50 mL
5.0 μm	50 mL
8.0 μm	50 mL
Coffee Filter	1L

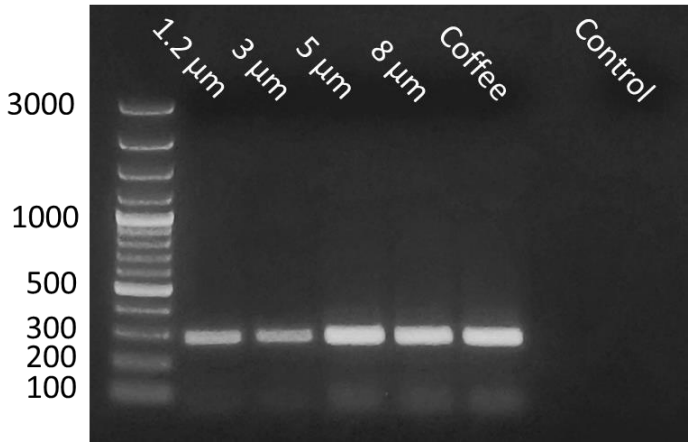

Figure 13. (A) Volumes of pond water filtered through the PCTE filters and coffee filter for concentration of eDNA samples. (B) Bands of expected size (276 bp) produced with primer set 5 through PCR amplification as compared to DNA ladder in 1.5% agarose gel electrophoresis were from filtrations across multiple PCTE filter sizes and Coffee filter with 1:1000 dilutions of aquarium water that contained newts with pond water. No template control on right.

From pond water dilutions, PCTE filter sizes between 8 µm and 1.2 µm clogged after filtering between 50 ml to 100 ml of pond water while the coffee filter and press was consistently able to filter > 1L of pond water for eDNA sampling (Figure 13A). Bands of expected size were produced by PCR amplification from all filter sizes (Figure 13B). Intensity was highest in the coffee filter extract and the 5 and 8 µm PCTE filter extracts and lowest in the 1.2 and 3 µm samples. The no-template control showed no visible band. Sequences from extracted bands matched target mitochondrial region for *N. meridionalis* (GenBank accession EU880322).

Nested PCR of clear and pond water trials using primer set 5.1a produced bands of expected size (not shown) and matched target mitochondrial sequence for *N. meridionalis* (GenBank accession EU880322). The no-template control showed no visible band.

Borescope Surveys

Sampling. Surveys were conducted between September 2015 and March 2018 at historical and predicted BSN locations (Figure 14, Appendix B) across the lower Rio Grande Valley (LRGV) identified by searching for ephemeral wetlands through Google Earth imagery, encompassing Cameron, Hidalgo, Willacy, and Starr counties with additional survey sites beyond the LRGV identified from literature (Rappole and Klicka 1991; Judd 1985). Some sites were also located through communications with the general public. Historical BSN sites identified through literature review included Laguna Atascosa National Wildlife Refuge (LANWR), Sabal Palm Sanctuary (SPS), Southmost Nature Conservancy (SMNC), Santa Ana National Wildlife Refuge (SANWR), Welder Wildlife Refuge, and the townships of Riviera and Vattmannville.

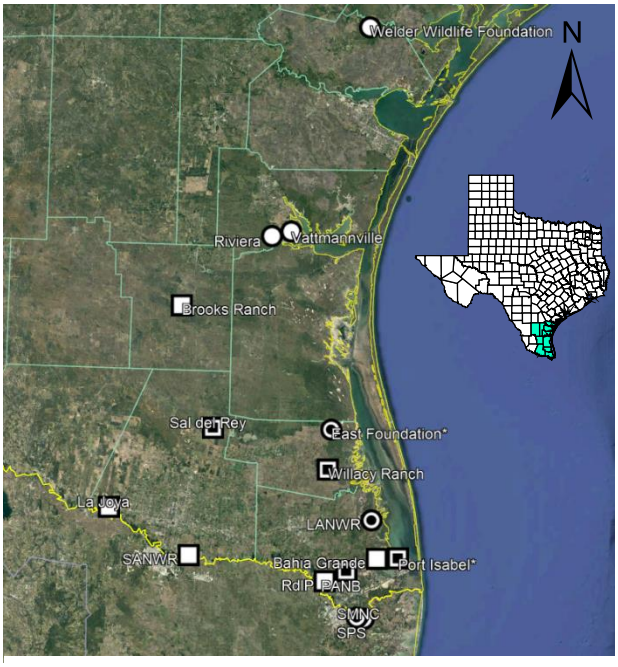

Figure 14. View of entire study region. Specific sites not depicted. Historical (circular) and undocumented (square) areas are identified, BSN individuals were found at filled markers. Four of nine sampled areas did not have historic records. Kenedy County (center) is almost entirely privately-owned ranchlands, access for biological research was not granted. Sites where samples were provided are identified with (*). Image provided by Google Earth.

Upon capture of each individual, location, snout-vent length (SVL), weight, and sex were recorded. DNA samples were taken from tail clips and ventral photographs were taken in case of recapture. All newts were handled under Institutional Animal Care and Use Committee (IACUC) approved protocols (2013-005-IACUC Kline). Special use permits for state and national parks were collected (PANB: PAAL-2016-SCI-0002; LANWR: 2016-23; SANWR: SA-06-15-16-CJP; and TPWD State Parks: SPR-0913-125). Surveys on private land were conducted following written landowner permission.

Surveying Methods. Visual inspections and debris searches were conducted at all field sites within 10 m of pond beds. Ponds were generally small (area < 1000 m^2, perimeter < 0.2 km) and sampling could be done around the entire perimeter. Cover boards were placed within dry pond beds and along pond banks at SMNC, Palo Alto National Battlefield (PANB), a private ranch in Willacy County, Resaca de la Palma State Park (RdlPSP), LANWR. and SPS. Two pitfall trap arrays of 100 m length with 5-gallon buckets placed every 20 m were constructed at SMNC and SPS.

At SMNC, Willacy, and PANB, burrows and fissures in pond beds and under cover boards were visually inspected with a borescope (Pyle Pvbor15; Figure 15A) for at least 5-min periods. The borescope camera stalk was marked every 1 cm with 1-cm wide strips of green duct tape being labeled every 10 cm, resulting in a striped pattern along the entire length with each cm marked by either tape or bare stalk for depth measurements. During borescope surveys, all animal encounters were photographed and upon visual observation of a newt, video was captured for remainder of cavity search. Borescope surveys could not be conducted at remaining sites due to the absence of cavities conducive to survey.

Figure 15. (A) Pyle Pvbor15 borescope used in this study with depth markings at every 1 cm, with 10 cm marks highlighted. B) Photo of BSN 5 cm down a fissure, found with borescope (Pyle Pvbor15). Multiple individuals were found with this strategy, some as deep as 35 cm underground.

Artificial burrows were constructed at Southmost Nature Conservancy (SMNC) along historical BSN habitat (Judd 1985; Rappole and Klicka 1991). Burrows were made by plunging a 4-cm diameter x 0.5-cm thick PVC pipe into the ground with a rubber mallet (Figure 16). The boring end of pipe was beveled inward to relieve pressure on exterior of pipe for easier extraction. Eight burrows were constructed, four were 0.5 m deep and the other four were 1 m deep. Paper tubes were placed in 0.5-m burrows as insets to allow moisture absorption and structure. Four 1-m long PVC pipes with perforations down the length and paper tube insets were placed in the 1-m deep burrows as added structure. Artificial burrows were surveyed with a borescope down the entire length and all animals encountered were photographed.

Figure 16. Artificial burrows were constructed at Southmost Nature Conservancy (SMNC) along historical BSN habitat (Judd 1985; Rappole and Klicka 1991). Burrows were made by plunging a 4-cm diameter x 0.5-cm thick PVC pipe into the ground with a rubber mallet.

Analysis. The total number of newt encounters were compared across survey techniques and refuges. Means and standard errors were calculated for SVL (cm) and weight (g), by sex. Regression analysis of SVL by weight was conducted. All analyses were conducted in Excel 2016.

Results.

Survey Techniques. Overall, 79 newts were recorded over the course of this study. Newts were found in five habitat types, two of which were heavily modified by human activity (Figure 17). Debris searches provided 57 accounts (72.2%), cover boards provided 12 (15.2%), borescope surveys resulted in eight (10.1%), and visual roving surveys yielded two (2.5%) (Figure 17). No newts were captured with pitfall traps surveys at two sites over a six-month period. Detailed assessments of each technique are provided below (Figure 17).

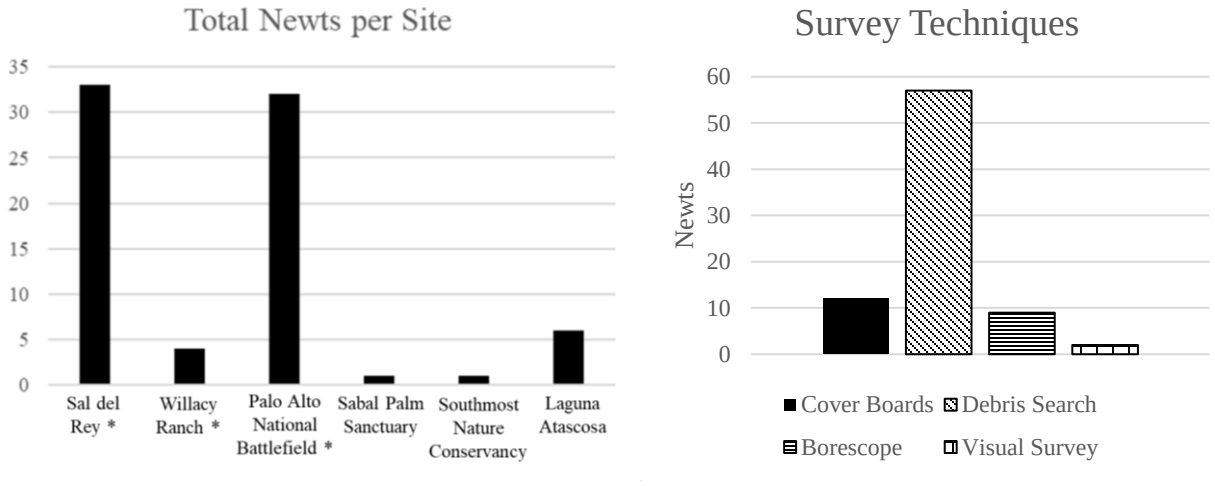

Figure 17. Total number of newts by site (left) and by technique (right). LEFT: Only six sites were found with newts during surveys, three of which had no previous documentation. Sal del Rey is an agricultural site where newts were found primarily under refuse tires. Newts reported herein from before surveying began (September 2015) are not included. RIGHT: Cover boards alone were only able to find 12 individuals, but when combined with borescopes were able to find 20 in total. Debris searches were the best strategy for finding newts, with a combined board and borescope technique coming second. Survey methods were generally associated with refuge types, with borescopes finding a majority of fissure dwelling individuals under boards. One borescope account was in an artificial burrow. Visual surveys produced very few accounts.

Debris searches provided the greatest number of newts. From PANB and the Sal del Rey site, the two most successful sites, 94.3% and 59.4% of newts were discovered through debris searches. In addition, all newts found during this study at Laguna Atascosa were found under debris, despite presence of cover boards.

Cover board surveys provided mixed results. In total, 12 newts were found directly under cover boards, but were limited to PANB (4 of 16 boards were successful) and the Willacy ranch (2 of 8 board clusters were successful). Boards from PANB were larger (1m x 1m x 4cm) but resulted in soil desiccation and fissuring underneath (Figure 18). Smaller boards of various sizes were placed at the Willacy ranch by the home owners in small clusters to cover a ~1 m^2 area. Borescope supplementation of cover boards surveys increased their effectiveness though, as described below.

Figure 18. Cover board with extreme soil desiccation underneath.

A total of eight newts were recorded using the borescope, none of which would have been detected without the ability to view into cavities. Seven of these sightings were made by viewing into fissures and burrows under traditional cover boards, while the last individual was found using a 0.5 m artificial burrow at SMNC. Newts discovered in this manner were between 5-cm and 50-cm deep, with some moving deeper upon discovery. Sightings at shallower depths (5 cm – 20 cm) were made a day or two after a rain event, some were discovered deeper (25 cm – 50 cm) after periods

Table 5. Habitat types with BSN presence during surveys.

Habitat Types
Sabal Palm Forest
Tamaulipan Thornscrub
Huisache Forest
Manicured Pasture
Agriculture Field

without rain. Across all sites where cover boards and borescopes were used, cover boards alone accounted for 12 captures and borescope surveys provided an additional eight, a 66% increase.

Along with newts, many other species were visually identified using borescopes in ground fissures and other cavities (*Table 6*). Borescope use was not applicable at all field sites nor under all conditions and could only be used when cavities were present. Densely vegetated areas generally had shallow cavities which were capable of visual inspection and sandy soil terrain did not form cavities.

Amphibians	**Invertebrates**
Notophthalmus meridionalis	Tarantula (*Aphonopelma* sp.)
Incilius valliceps	Ants (Formicidae)
	Millipede (Diplopoda)
Reptiles	Centipede (Chilopoda)
Unidentified snake	Cricket (Gryllidae)
	Snails and Slugs (Gastropoda)
Mammals	Fiddler Crab (*Uca* sp.)
Neotoma micropus	

Table 6. List of animals found in cavities or fissures. Potential predators and prey for the BSN were found in these locations. Argentine ants were found, an invasive species that may kill newts.

BSN Size and Length. The relationship for SVL and weight showed the same pattern for both sexes (Fig. 10). However, females were captured at larger sizes than males and were found to have a greater range in size and weight compared to males (Figure 19). The largest caught female was 9.5 g and 6.97 cm SVL, compared to the largest male measurements of 3.8 g and 5.3 cm SVL. Females weighed an average of 4.1 g (± 0.4) with an SVL averaging 5.10 cm (±0.11), while males were smaller at an

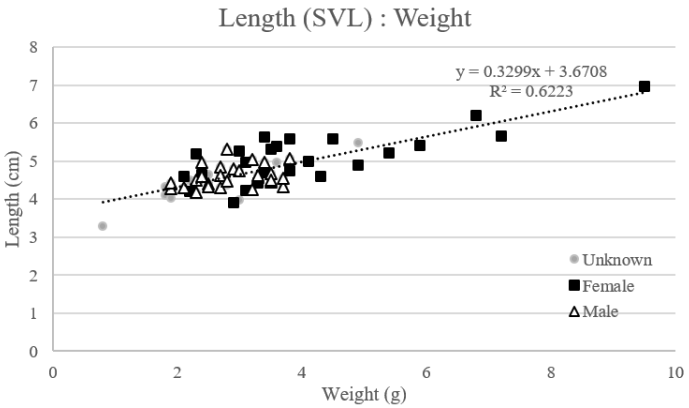

Figure 19. Weight : length (SVL) measurements based on sex. Females and males were assessed based on characters described by Mecham (1968b).

average weight of 2.9 g (± 0.1) and SVL of 4.59 cm (±0.05). Females ranged from 2.1 g to 9.5 g with SVL between 3.91 cm and 6.97 cm; males had considerably smaller ranges from 1.9 g to 3.8 g in weight, and 4.18 cm to 5.32 cm SVL. Total weight range across all newts captured was from 0.8 g to 6.97 g, and total SVL ranged from 3.3 cm to 6.97 cm. The sex ratio was roughly even with 24 females being found compared to 28 males, sexes for 27 individuals could not be determined.

Discussion

In this study an assay was developed for BSN detection in pond water conditions prevalent in south Texas and was successful at identifying BSN DNA at a 1:1,000 dilution. The BSN5 and nested BSN5.1 primer sets used with pond water filtered with off-the-shelf coffee press and filters shows promise as a future wetland survey technique for black-spotted newts. While PCTE filters were able to detect BSN DNA in pond water trials (Figure 13), coffee filter trials provided equivalent results, with the capacity of filtering more water using a simpler technique with less expensive materials. The ability to detect newts in water bodies without need for physically capturing them, will greatly reduce time, effort, and cost in monitoring efforts (Cowart, Murphy, and Cheng 2017; Sigsgaard et al. 2015). Additionally, the sensitivity of eDNA assays provides a greater probability of detection over traditional techniques (Williams et al. 2017; Furlan et al. 2016), a necessity when studying cryptic species. Primer set 5 was capable of detection of BSN DNA in spiked pond water trials. The additional use of primer set 5.1a in nested PCR reactions can be used to detect and directly sequence samples at even lower concentrations than assessed in this study.

Resaca water in the LRGV is highly turbid, resulting in rapid filter clogging of smaller pore sizes even when using vacuum suction, which reduces eDNA collection. Many eDNA studies have reported filtering greater than 2 L of water through filters with 1.2 µm pore size, or less

(Wilcox et al. 2015; Katano et al. 2017; Agersnap et al. 2017; Davy, Kidd, and Wilson 2015). These studies were often conducted in flowing streams (Katano et al. 2017; Kuppert 2015; Goldberg et al. 2011) or lakes (M. R. Williams et al. 2017; Tessler et al. 2017; Veldhoen et al. 2016; see review in Thomsen and Willerslev 2015). Comparatively, water bodies within the LRGV are typically low-turnover systems and are periodically filled by runoff from agricultural fields (Jahrsdoerfer and D. M. Leslie 1988) or become highly turbid by fine particulate disturbance (Rappole et al. 1986). The assay developed herein is a significant improvement in eDNA monitoring from eutrophic and turbid waters.

Advancements in eDNA technology also show promise of even greater applications in ecological research. Degradation rates of eDNA are influenced by a multitude of factors including temperature, UV-B, and bacterial presence (Strickler, Fremier, and Goldberg 2015; Tsuji et al. 2017; Goldberg, Strickler, and Pilliod 2015). While degradation rates have not been studied in South Texas systems, high temperatures and UV exposure of the region could limit eDNA persistence. Consequentially, future studies could incorporate BSN detections on a temporal scale (Minamoto et al. 2017; Sassoubre et al. 2016; Bista et al. 2017; Guardiola et al. 2016), providing estimates for newt migration out of breeding ponds. Work with bighead carp found greater abundance of target eDNA in warm waters compared to cooler waters nearer the water's edge (Takahara et al. 2012), indicating assessment of spatial use is also possible in aquatic settings with behavioral and ecological implications. Alternatively, local genetic variation may be assessed using primer sets targeting genes of higher variability (Bakker et al. 2017; Uchii et al. 2017), allowing estimates of a population's genetic variation prior to more rigorous genetic testing.

For terrestrial newts, borescope surveys tested in this study were shown to be an effective supplement to standard survey techniques. Fissures and burrows are common sights in the clay

pan bottom of dried pond beds in the LRGV, offering refuge for black-spotted newts and other species. Traditional cover board surveys were enhanced with the use of the borescope because of soil fissuring underneath. Additionally, borescope surveys revealed many cohabitants, including potential prey and predators, similar to camera survey results of gopher tortoises (*Gopherus* spp.) (Smith et al. 2005; Butler and Harris 2010). Borescope findings revealed a substantial underground ecology which deserves continued monitoring and assessment.

Traditional terrestrial amphibian survey techniques are capable of detecting newts but succumb to various problems. Cover boards provide significant refuge above ground but prohibit moisture from penetrating covered areas. Without moisture, covered terrain became jagged and fractured over time due to continued drying, making cover boards less effective after a year. Only at Palo Alto National Battlefield and the Willacy County ranch, were cover boards ever found with newts. Cover boards have repeatedly been found to have limited results with terrestrial salamanders (G. F. Strain, Raesly, and Hilderbrand 2009; Gabriel F. Strain and Raesly 2006; Mitchell, Erdle, and Pagels 1993; McDade and Maguire 2000), with pitfall traps being the more preferred method. While cover boards were initially effective at some sites, especially in conjunction with borescopes, fissures that eventually developed became unmanageable and made the borescope strategy more difficult as fissures grew in depth and width. In future surveys, the use of larger cover boards with small perforations or overlapping smaller boards may remedy the fissure dilemma by allowing moisture underneath, thereby limiting fissure growth.

Pitfall trap arrays are often used successfully for salamander surveys however, they were unsuccessful in this study and problems have been reported in a previous BSN study. Rappole and Kicka (1991) reported individuals under the traps and in fissures formed along drift fences rather than in the traps themselves and this study was unsuccessful at finding any newts with pitfall traps.

Soil and climate parameters where newts are found make pitfall traps effectively useless, as fissures develop in the heat wherever soil is split. Additionally, limited dispersal capabilities (Rappole and Klicka 1991) and the habit of seeking refuge underground (Bare and Kline 2017) only aggravates the situation. Comparatively, borescope technology takes advantage of cavities that provide refuge for newts, turning cavities into passive traps that can be monitored. This, in conjunction with artificial burrows detailed in this study shows promise to increase detections of black-spotted newts.

The results of the present study suggest that small footprint debris are ideal habitat for newts. When newts were found under debris, it was typically with thin footprints no more than 20 cm wide. At PANB, newts under debris were mostly under pieces of bark, but small branches were also effective with one newt even being found in the root system of a dead huisache tree. Similarly, at Laguna Atascosa newts were primarily found under small fallen branches, with one exception being found among the roots of a dead tree. While newts were not found in Vattmannville, a local rancher did detail personal newt encounters after uprooting multiple trees. Comparatively, multiple abandoned tires along a slight incline in Hidalgo County yielded the most newts observed at any site during this study. Tires provided sufficient cover with a rimmed edge allowing for greater moisture penetration directly underneath and are heavy enough to sink into the ground to create a moisture barrier, protecting against desiccation. Since tires may leach chemicals and pose risks to salamanders, replacement of tires with an alternative cover that can mimic these parameters is suggested. Other sites such as SMNC and SPS were densely vegetated, providing continual leaf litter coverage across the site potentially negatively impacting the results of cover board surveys. Moreover, findings to suggest cavity use, especially along tree root systems, indicates that densely

vegetated areas provide substantial terrestrial BSN microhabitat exacerbating an already difficult search.

Additional surveying techniques described herein can significantly increase BSN detection rate for. These methods are simple, inexpensive, and passive allowing for more efficient data gathering with minimal intrusion. Use of eDNA can facilitate landowner cooperation because it reduces the need for land disturbance and raises the potential for regional citizen science (Biggs et al. 2015). The cryptic nature of the species has made it difficult to monitor for previous studies. Development of more sophisticated and sensitive techniques for BSN detection has been sorely lacking, until now. These strategies are not restricted solely to black-spotted newts and may be applied to other studies of cryptic herpetofauna in the Tamaulipan Biotic Provence and elsewhere. With refinement, both survey methods detailed here may be used in a focused manner on single species or on an ecosystem scale to study whole community structure.

CHAPTER IV

POPULATION GENETICS: CONSERVATION IMPLICATIONS FOR THE BLACK-SPOTTED NEWT

Introduction

An understanding of population structure and connectivity is of utmost importance for proper conservation assessments and policy decisions. High levels of habitat fragmentation and landscape modification can have great impacts on population connectivity (Greenwald, Gibbs, & Waite, 2009; Greenwald, Purrenhage, & Savage, 2009; Kolozsvary & Swihart, 1999; Noël, Ouellet, Galois, & Lapointe, 2007), especially in species with low dispersal rates (Cushman, 2006; Greenwald, Purrenhage, et al., 2009; Sawyer, Epps, & Brashares, 2011). Even minor changes to the natural environment can have cascading effects on a local population's stability, leading to increased fragmentation over the course of years and, possibly, population reduction or local extinction (Liselotte W. Andersen, Fog, & Damgaard, 2004; Cosentino et al., 2014; Cushman, 2006; Dixo, Metzger, Morgante, & Zamudio, 2009). Environmental degradation can have an even greater impact on already small populations, altering demographic ratios by impacting specific life stages or life history events (Mullen, Woods, Schwartz, Sepulveda, & Lowe, 2010; Roe & Grayson, 2008).

Small or genetically isolated populations may suffer secondarily through genetic drift and inbreeding depressions, leading to reduced genetic heterozygosity and variability (Janecka et al.,

2016; Noël et al., 2007). Inbreeding depressions have been linked to negative impacts on demography (L. W. Andersen, Fog, & Damgaard, 2004; Frankham, 1995; Storfer, Mech, Reudink, & Lew, 2014), shifting population structures out of balance and causing a decline in population. Moreover, reduced genetic variability negatively impacts population adaptability to environmental changes (Johansson, Primmer, & Merilä, 2007).

The Black-spotted Newt (*Notophthalmus meridionalis*, BSN) is a relatively unknown species in the literature (Judd, 1985; Mecham, 1968a; Rappole & Klicka, 1991). This species occurs in the Tamaulipan thornscrub and Gulf Coast Prairies ranging along the Mexican Gulf Coast from San Patricio County, Texas to central Veracruz, Mexico. Two distinct subspecies of BSN are recognized based on morphological characters (Mecham, 1968b). The species is listed as Globally Endangered (IUCN), with the Mexican government also listing it as Endangered (SEMARNAT, 2010) and Texas listing it as Threatened (Texas Parks and Wildlife Department, 2000). Comprehensive genetic study is needed across the range to determine population variability and potential impacts of genetic isolation due to habitat fragmentation. In addition, no genetic study to date has included multiple source data or samples from the endemic Mexican subspecies (*N. m. kallerti*).

A previous BSN study identified localized metapopulations (Rappole & Klicka, 1991), networks of spatially discrete populations (i.e., subpopulations) linked by dispersal, strictly based on regional captures and relying on an assumption of life stage similarity to the Eastern Newt (*N. viridescens*), a sister species. Over the course of the past century, human encroachment in the Lower Rio Grande Valley (LRGV) has drastically increased habitat fragmentation (Jahrsdoerfer & D. M. Leslie, 1988). Genetic work including *N. meridionalis* has focused primarily on congeneric relations, and used only individual samples (Reilly, 1990; Weisrock et al., 2006).

Without a greater understanding of population genetic variation, no assessments have been made regarding connectivity among populations or degree of regional isolation across the range.

Mitochondrial sequences can be used to assess population connectivity. Much of the mitochondrial genome is coding DNA, making it more highly conserved than non-coding microsatellites (Bazin, 2006) and maternal origins of mitochondrial DNA offers a valuable control mechanism for assessing connectivity (Dyer & Nason, 2004; Whitmore, Losee, Meyer, & Spradling, 2013; Zardoya & Meyer, 1998). Furthermore, lack of cross-over in mitochondrial DNA reduces inter- and intra- variability within a metapopulation ensuring higher sequence consistency for a population and simpler analysis of genetic relatedness among populations.

This study is the first to compare genetic data across multiple sources or isolate genetic material from the southern subspecies (Mexico BSN). The aims of this study are to 1) investigate genetic variability of BSN populations within the LRGV of Texas, 2) compare genetic distances between BSN subspecies, and 3) estimate time of divergence of the BSN subspecies.

Methods

Field Sites

Field sites were located within the LRGV of South Texas (Figure 20) including Cameron, Hidalgo, and Willacy counties. Historical records were used to determine high priority target areas including Laguna Atascosa National Wildlife Refuge (LA), Southmost Nature Conservancy (SMNC), and Sabal Palm Sanctuary (SP). Newts were also sampled at Palo Alto National Battlefield (PA), along the public right-of-way in Hidalgo County, and private property in Willacy County. More sites in the LRGV were visited where newts were not encountered.

Sampling

Cover board surveys, debris checks, pitfall trap arrays, and borescope surveys (see Chapter III) were conducted to collected newt samples (Figure 20), with global positioning systems (GPS) coordinates taken for each collection. Tail clippings ~1 cm long were preserved in a 70% ethanol solution and stored in a -20^{o} C freezer. Ventral photographs were taken for individual identification, ensuring that inadvertent resampling did not occur. All newts were handled in accordance to Institutional Animal Care and Use Committee (IACUC) protocols (2013-005-IACUC Kline) and collections were conducted under Texas Parks and Wildlife Scientific Collecting Permit (permit SPR-0913-125).

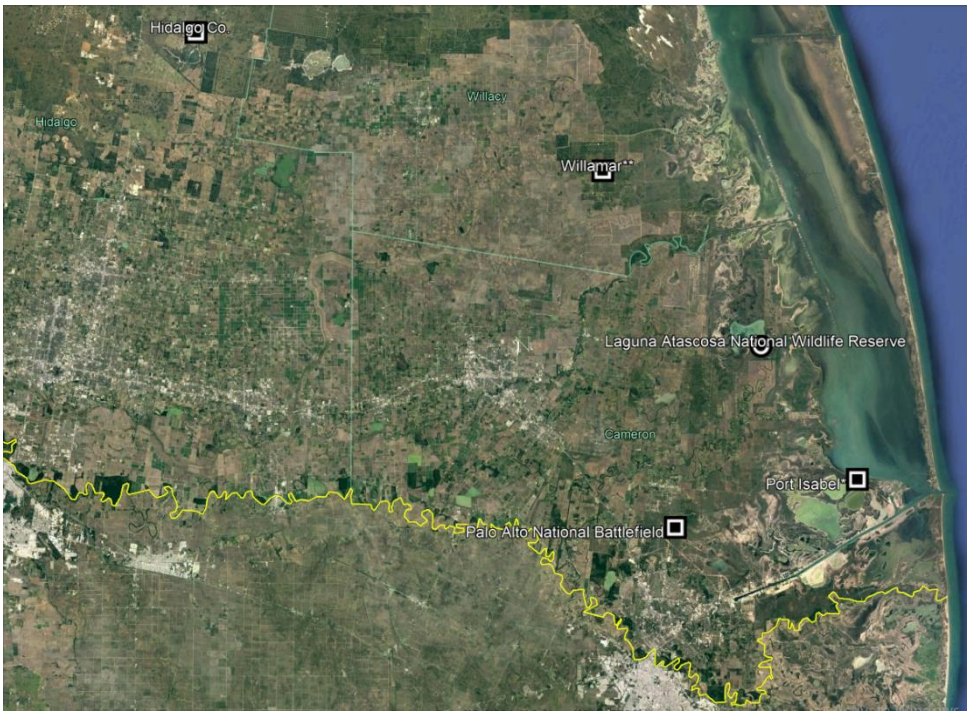

Figure 20. Sampling sites used in this study. Locations have been buffered to conceal exact locations. Port Isabel samples (*) were provided by Seth Patterson. A Willamar sample (**) was amplified but failed to sequence for unknown reasons. Two samples were sequenced from Port Isabel and Laguna Atascosa National Wildlife Reserve, and three from the Hidalgo County site. Three samples were sequenced from Altamira, Tamaulipas, Mexico (not shown). Laguna Atascosa locations had been documented before this study (circle), all others are new locations (square).

Laboratory Work

Extraction of BSN DNA from ethanol preserved tissue was done using the GenCatch Blood & Tissue Mini-Prep Kit (#1460050), Epoch Life Science Sugar Land, TX, USA) using the mouse tail protocol. Polymerase chain reaction (PCR) was conducted to amplify mitochondrial DNA in three overlapping regions of expected size 5543 bp, 5723 bp, and 5792 bp (Figure 22). Each amplification was conducted using 25 µL reactions with GoTaq Long Master Mix (Promega Corp, Madison, WI), 20 ng of DNA template, and BSN specific primers (Table 7 and Table 8). A 5µL aliquot of the PCR reaction was run on 0.7% agarose TBE gel to confirm successful amplification. The remainder was extracted with a PCR cleanup kit (GenCatch PCR Cleanup Kit #2360050, Epoch Life Science Sugar Land, TX, USA). Initial purity and concentrations were measured using a Nanodrop. All three amplified products were diluted to a 0.2 ng/µL concentration measured by Qubit fluorometer (ThermoFisher Scientific, Q33216) and then subsampled to form a 0.2 ng/µL mixture using equal amounts of each region to represent the full mitochondrial genome of individual newts and remeasured with Qubit. Combined fragment mixtures were sent to Harvard Medical School Biopolymers Lab for library preparation and Illumina Nextera sequencing.

Table 7. All primer sets tested for this study. Full mitochondrial genomes were constructed using primer sets GTOL, L1C2, and C2G.

Primer Set	***Primer***	***Sequence***	***Amplicon Length***
GTOL	BSN-K9FW-GTOL1	TCTATCTACGCACCGAGAAGG	5543
	ALL-K10RV-GTOL1	CATCCCACTCTTTTGCCACAG	
L1C2	BSN-L1-FW1	ACACCGCCCGTCACCCTCT	5723
	BSN-C2-RV2	CAACATTTCCTTGGACTTGCCG	
C2G	BSN-C2-FW1	TGTTTTATCTATTGGGGCTGTATTCG	5792
	BSN-G-RV-2INT	TGATGGGATGGTGATACGCC	

Table 8. Polymerase chain reaction (PCR) conditions for each PCR conducted for this study. GTOL, C2G, and L1C2 amplified individual trits of entire mitochondria (see Figure 22), eDNA sets indicated. All mixtures conducted with GoTaq Long MasterMix.

Primer Set	*Primer Vol (@ 10 uM)*	*Amount of Template*	*Cycles*	*Dissociation Phase*	*Annealing Phase*	*Extension Phase*
GTOL	1 uL	5 - 20 ng	37	30 s @ 94 C	30 s @ 56 C	7 min @ 65 C
C2G	1 uL	5 - 20 ng	37	30 s @ 94 C	30 s @ 58 C	7 min @ 65 C
L1C2	1 uL	5 - 20 ng	37	30 s @ 94 C	30 s @ 64 C	7 min @ 65 C

Sequence Processing and Assembly

Nextera libraries were imported into Geneious 11.0.1 (Kearse et al., 2012) where they were trimmed, removing Illumina primer ends with the BBTrim function (Bushnell, 2017) and pairs merged using BBMerge (Bushnell, 2017) at the lowest sensitivity settings. Merged sequences under 140-bp were removed. Trimmed and merged libraries were mapped to GenBank reference sequence EU880322 (*N. meridionalis*) using the Geneious Map to Reference function with three iterations at Med-High sensitivity.

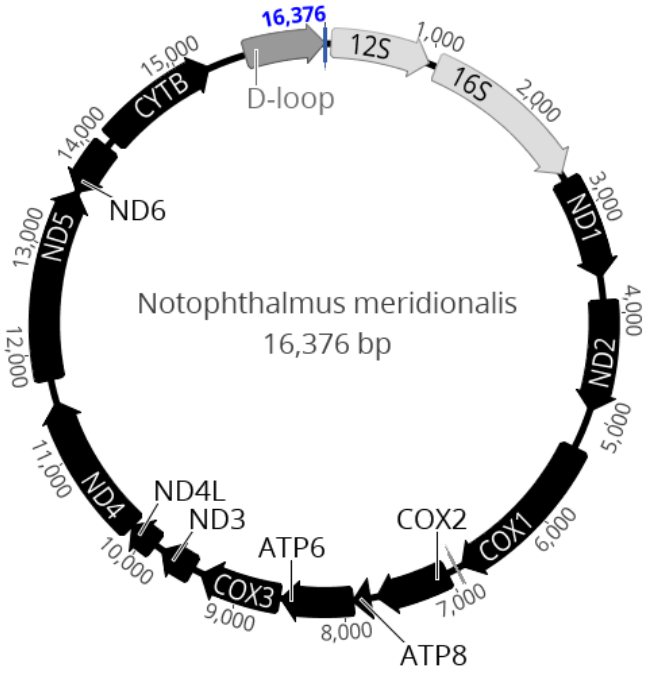

Figure 21. *N. meridionalis* mitochondrial genome representation. Protein coding regions highlighted in black, rRNA genes in light grey, and D-loop in dark grey. Clockwise directed genes are on the heavy strand and counter-clockwise directed genes are on the light strand.

Consensus sequences were based on majority nucleotide at each location with annotations transferred from EU880322 (Figure 21). Protein coding region annotations were manually

assessed to ensure protein sequences began and ended with appropriate start and stop codons respectively.

Mitochondrial Sequence Analysis

Mitochondrial sequences were aligned with GenBank sequences for each *Notophthalmus* species – *N. meridionalis* (EU880322), *N. viridescens* (EU880323), and *N. perstriatus* (EU880323) - and *Taricha rivulosa* (EU880334) as an outgroup from the sister genus. Two alignments were generated: full mitochondrial sequences and partial sequences. A total of 35 genes (two rRNA, 13 protein coding, 20 tRNA genes, and the D-Loop) were extracted from sequences and concatenated for sequence analysis. Two tRNAs (Asp and Lys) were removed from full mitochondrial analysis due to incompleteness of *T. rivulosa* (GenBank accession EU880334). Regions amplified by the primer set C2G (Table 7 and Figure 22) and part of the D-Loop were removed from partial sequence analysis due to PCR failure of C2G region in two Mexico samples and incomplete sequence of the D-Loop in the BSN GenBank sequence (EU880322). Percent dissimilarities, and number of nucleotide and amino acid changes were identified across all unique haplotypes.

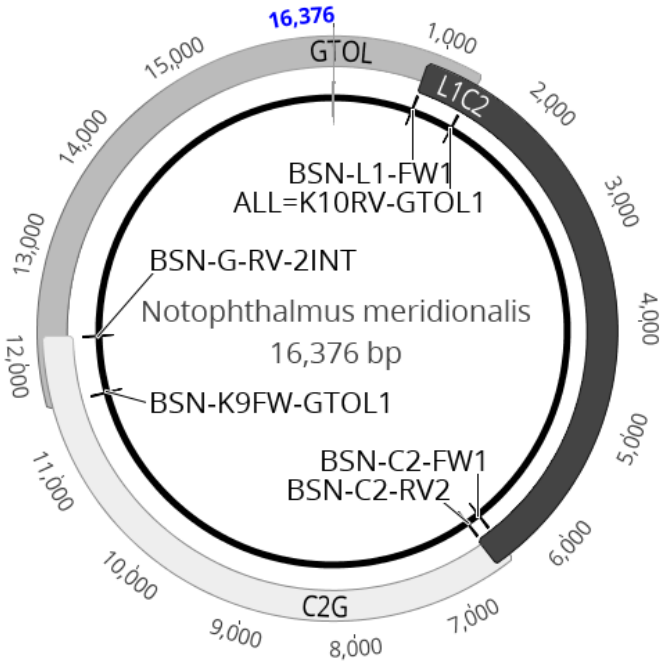

Figure 22. Representation of each PCR amplified trit of mitochondrial genome including primer locations. Each over hang is at least 100-bp for reference during sequence building in Geneious.

A Mantel test was conducted using the partial mitochondrial sequence dataset and mantel function from the VEGAN package (Dixon, 2003) for R with partial sequence alignments for genetic dissimilarity and geographic distance matrices using 9999 bootstrap replicates in R-3.3.3.

Amino acid shifts for all successfully sequenced coding regions were assessed manually using the onboard Geneious vertebrate mitochondrial DNA amino acid translation.

Model schemes for full and partial alignments were assessed using the PartitionFinder (Lanfear, Calcott, Ho, & Guindon, 2012; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017) greedy algorithm (Guindon et al., 2010) with each gene and codon position as separate partitions. Schemes with the lowest Akaike Information Criterion score with correction for small sample sizes (AICc) were used for further analysis. Phylogenies using both partial and complete sequence alignments were generated using partitions and models specified by PartitionFinder and directly compared. Maximum Likelihood analysis (MLA) was conducted using the RAxML (Stamatakis, 2006) Geneious plugin (RAxML Vers. 3.0, Biomatters Ltd.) with 1000 bootstraps using partitions and substitution model specified from PartitionFinder. MrBayes 3.2 (Ronquist et al., 2012) was used to run three Bayesian Analyses in parallel for one million generations and a burn-in value of 0.25. Bayesian Analyses were based on substitution models specified by PartitionFinder.

Timeline Divergence Analysis

Following the procedure described by Whitmore et al. (2013), concatenated sequences for the Cytochrome B (CytB), D-Loop, and tRNA-Pro genes were compiled with the same regions from Whitmore et al. (2013) (GenBank Accessions JX570765-JX570781). Sequences were aligned using the MUSCLE algorithm and the phylogeny was produced using RAxML with a GTR+I+G model with 1000 bootstraps. Percent identity values across BSN subspecies samples were used to estimate a divergence timeline based on values provided in Whitmore et al. (2013).

Table 9. Gene sequences for mitochondrial genome PartitionFinder analysis. Full genome (left) and partial genome (right). Asp and Lys are not available for outgroup *Taricha rivulosa* (GenBank Accession 880334) and are not included in full sequence analysis.

Synthesis	Gene	Location
rRNA	12S rRNA	1-948
rRNA	16S rRNA	949-2534
Protein	ATP6	2535-3218
Protein	ATP8	3219-3386
Protein	COX1	3387-4937
Protein	COX2	4938-5634
Protein	COX3	5635-6471
Protein	CYTB	6472-7647
Non-coding	D-Loop	7648-8454
Protein	ND1	8455-9423
Protein	ND2	9424-10467
Protein	ND3	10468-10815
Protein	ND4	10816-12246
Protein	ND4L	12247-12543
Protein	ND5	12544-14358
Protein	ND6	14359-14877
tRNA	Ala	14878-14946
tRNA	Asn	14947-15019
tRNA	Asp	15020-15089
tRNA	Cys	15090-15155
tRNA	Gln	15156-15228
tRNA	Glu	15229-15296
tRNA	Gly	15297-15365
tRNA	His	15366-15434
tRNA	Ile	15435-15507
tRNA	Leu1	15508-15582
tRNA	Leu2	15583-15652
tRNA	Met	15653-15721
tRNA	Phe	15722-15791
tRNA	Pro	15792-15864
tRNA	Ser1	15865-15935
tRNA	Ser2	15936-16003
tRNA	Thr	16004-16071
tRNA	Trp	16072-16140
tRNA	Tyr	16141-16208
tRNA	Val	16209-16277

Synthesis	Gene	Location
rRNA	12S rRNA	1-948
rRNA	16S rRNA	949-2534
Protein	COX1	2535-3815
Protein	CYTB	3816-4991
Non-coding	D-Loop	4992-5705
Protein	ND1	5706-6674
Protein	ND2	6675-7718
Protein	ND5	7719-9533
Protein	ND6	9534-10052
tRNA	Ala	10053-10121
tRNA	Asn	10122-10194
tRNA	Cys	10195-10260
tRNA	Gln	10261-10333
tRNA	Glu	10334-10401
tRNA	Ile	10402-10474
tRNA	Leu1	10475-10549
tRNA	Leu2	10550-10619
tRNA	Met	10620-10688
tRNA	Phe	10689-10758
tRNA	Pro	10759-10831
tRNA	Ser2	10832-10892
tRNA	Thr	10893-10960
tRNA	Trp	10961-11029
tRNA	Tyr	11030-11097
tRNA	Val	11098-11166

Table 10. Best scheme described by PartitionFinder for full mitochondrial sequence (InL = -38603.3204346, AICc = 77784.7634304). Codon base pair positions for protein coding genes are indicated by superscript value.

Subset	*Best Model*	*# Sites*	*Partitions*
1	GTR+G	3325	12S, 16S, COX2[1], Asp, Gly, His, Ile, Leu2, Met, Phe, Ser2
2	GTR+I	2844	ATP8[1], ATP8[2], ATP6[1], CYTB[1], ND1[1], ND2[1], ND3[1], ND4[1], ND4L[1], ND5[1], Leu1, Val
3	HKY+G	1873	ATP6[2], ND2[2], ND3[2], ND4[2], ND4L[2], ND5[2]
4	GTR+I+G	2355	ATP6[3], ATP8[3], COX2[3], COX3[3], CYTB[3], ND2[3], ND3[3], ND4L[3], ND5[3]
5	GTR+I	796	COX1[1], COX3[1]
6	HKY+I	1743	COX1[2], COX2[2], COX3[2], CYTB[2], ND1[2]
7	GTR+G	1317	COX1[3], ND1[3], ND4[3]
8	HKY+G	875	D-Loop, Thr
9	HKY+G	561	ND6[1], ND6[2], Ala, Gln, Pro
10	HKY+I	173	ND6[3]
11	HKY	415	Asn, Cys, Glu, Ser1, Trp, Tyr

Table 11. Best scheme described by PartitionFinder for partial mitochondrial sequence (InL = -29587.5675049, AICc = 59774.8723408). Codon base pair positions for protein coding genes are indicated by superscript value.

Subset	*Best Model*	*# Sites*	*Partitions*
1	GTR+G	4544	12S, 16S, CYTB[1], ND1[1], ND2[1], ND5[1], Ile, Met, Phe, Ser2,Val
2	GTR+I	773	COX1[1], Asn, Cys, Leu2, Trp, Tyr
3	HKY+I	2343	COX1[2], CYTB[2], ND1[2], ND2[2], ND5[2], ND6[2], Leu1
4	GTR+G	427	COX1[3]
5	GTR+I+G	1668	CYTB[3], ND1[3], ND2[3], ND5[3]
6	HKY+I	855	D-Loop, Pro, Thr
7	HKY+I	383	ND6[1], Ala, Gln, Glu
8	GTR	173	ND6[3]

Table 12. Nucleotide dissimilarity matrix of full mitochondrial genome haplotypes. GenBank accessions for *N. viridescens* (EU880323), *N. perstriatus* (KP013091), and *Taricha rivulosa* (EU880334) used for comparison. Percent dissimilarity (below diagonal) and number of differences (above diagonal) represented. Haplotype KAL2 not represented due to amplification failure of L1C2 mtDNA amplicon.

	MER1	**MER2**	**MER3**	**MER4**	**MER5**	**KAL1**	**EU880323**	**KP013091**	**EU880334**
MER1		12	26	20	28	337	1410	1570	2813
MER2	0.074		26	20	28	338	1411	1570	2813
MER3	0.161	0.161		16	26	339	1416	1577	2822
MER4	0.124	0.124	0.099		22	331	1410	1569	2808
MER5	0.173	0.173	0.161	0.136		334	1412	1568	2812
KAL1	2.084	2.090	2.095	2.047	2.065		1382	1565	2815
EU880323	8.714	8.721	8.748	8.714	8.727	8.541		1241	2744
KP013091	9.705	9.705	9.745	9.699	9.693	9.674	7.672		2842
EU880334	17.317	17.317	17.366	17.286	17.311	17.327	16.886	17.490	

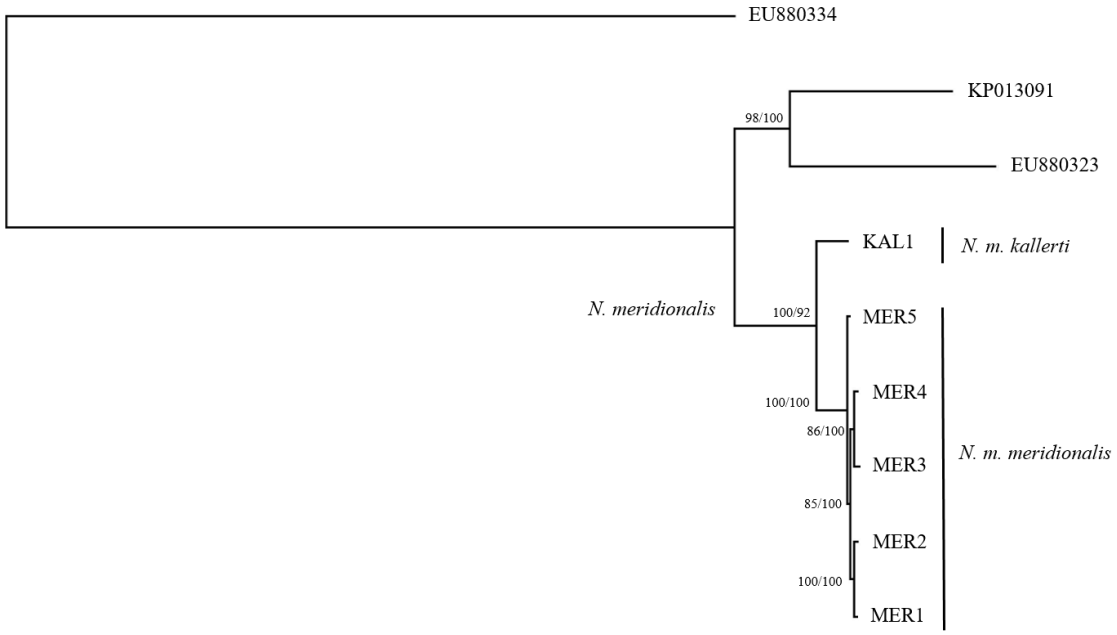

Figure 23. Full mitochondrial genome phylogeny. Maximum likelihood bootstrap values (top) and Bayesian confidence percentages (bottom) represented. GenBank sequences: *Taricha rivulosa* (EU880334), *Notophthalmus perstriatus* (KP013091), and *N. viridescens* (EU880323). GenBank sequence for *N. meridionalis* (EU880322) not used due to incomplete regions.

Table 13. Nucleotide dissimilarity matrix of partial mitochondrial genome haplotypes. GenBank accessions for *N. meridionalis* (EU880322), *N. viridescens* (EU880323), *N. perstriatus* (KP013091), *Taricha granulosa* (EU880333), and *T. rivulosa* (EU880334) used.

	MER1	**MER2**	**MER3**	**MER4**	**MER5**	**KAL1**	**KAL2**	**EU880322**	**EU880323**	**KP013091**	**EU880333**	**EU880334**
MER1		7	19	12	20	199	202	14	864	965	1767	1650
MER2	0.067		18	11	19	200	203	13	866	966	1769	1649
MER3	0.183	0.173		11	19	202	205	13	871	971	1771	1654
MER4	0.115	0.106	0.106		14	195	198	6	865	963	1764	1645
MER5	0.192	0.183	0.183	0.135		197	200	16	869	964	1769	1650
KAL1	1.912	1.922	1.94	1.874	1.893		7	197	848	961	1750	1656
KAL2	1.941	1.951	1.969	1.903	1.922	0.067		200	851	964	1751	1659
EU880322	0.135	0.125	0.125	0.058	0.154	1.893	1.922		865	966	1768	1649
EU880323	8.299	8.318	8.361	8.309	8.347	8.145	8.174	8.309		764	1714	1617
KP013091	9.271	9.28	9.323	9.252	9.261	9.232	9.26	9.28	7.337		1774	1663
EU880333	16.948	16.967	16.977	16.919	16.967	16.782	16.791	16.958	16.433	17.01		993
EU880334	15.829	15.819	15.858	15.781	15.829	15.883	15.912	15.819	15.505	15.949	9.523	

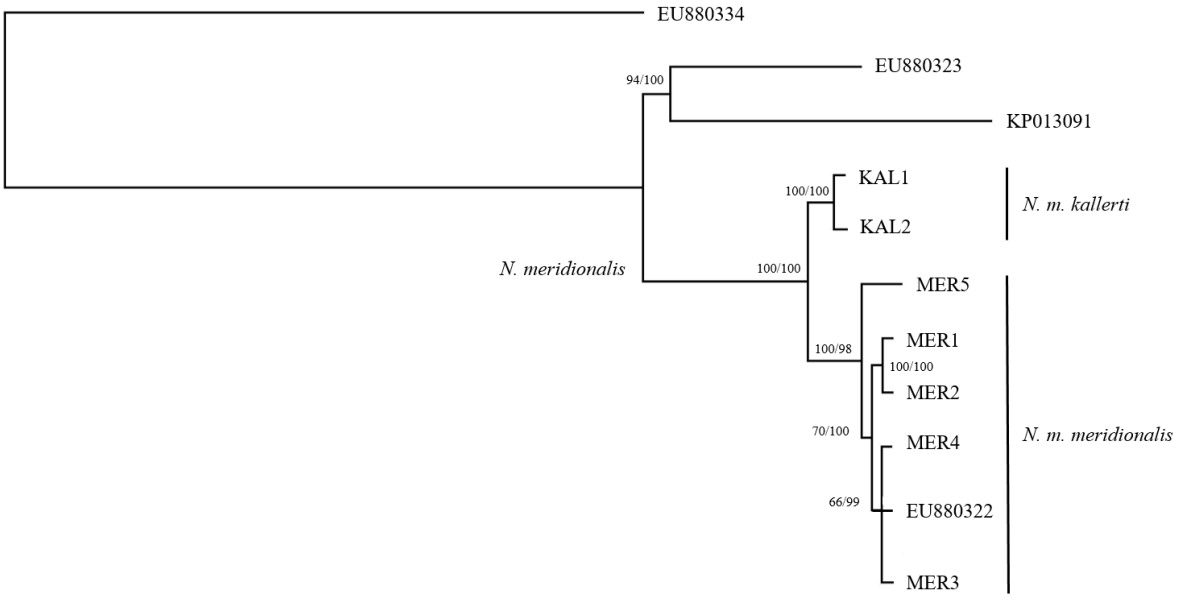

Figure 24. Partial mitochondria phylogeny. Maximum likelihood bootstrap values (top) and Bayesian confidence percentages (bottom) represented at nodes. Phylogeny adds haplotype KAL2 and *N. meridionalis* accession (EU880322) compared to the full mitochondria phylogeny (Figure 23). GenBank accession (EU880322) is derived from individual housed at Gladys Porter Zoo with unknown origin, it is grouped here with haplotypes from Laguna Atascosa National Wildlife Refuge (MER4) and Hidalgo County (MER3). All *N. meridionalis* haplotypes form monophyletic clade with subspecies forming monophyletic subclades.

Results

The total number of reads for successful Nextera runs ranged from 1,108,476 to 6,200,710 with an average read number of 2,180,892 (Appendix C). Consensus sequence coverage averaged above 2000 reads across all successful samples. D-loop coverage was consistently lower, dropping as low as 19 coverage.

Seven unique haplotypes, five from Texas samples (MER1-5) and two from Mexico samples (KAL1-2), were found across twelve samples sent for sequencing. One sample failed (Willamar) during Nextera sequencing. Hidalgo County samples (haplotype MER3) all shared a 6-bp duplication within the 12S rRNA gene at position 885. Mexico haplotypes (KAL1-2) shared an insertion event in the 16S rRNA gene at position 603 and a deletion at position 5,160 in an untranslated region but were differentiated by two amino acids in the ND5 protein. Across all haplotypes, 61 amino acid (AA) variations were identified, 20 of which were unique only to Mexico. MER1-5 varied by fewer than ten AA from each other, while KAL1 (only complete Mexico haplotype) diverged by 48-50 AA compared to MER1-5 (Table 14). Compared directly to *N. viridescens* (EU880323), MER1-5 varied by 157-160 AA while KAL1 differed by 145 AA. Across all complete *Notophthalmus* mitochondrial sequences, KAL1 shared 25 AA differences from consensus with *N. viridescens*, 17 of which were shared with *N. perstriatus* as well. Comparatively, only 14 changes were shared between Texas BSN and *N. viridescens*.

Partial mtDNA Mantel test was nonsignificant ($p > 0.5$) showing no correlation between genetic and geographical distances. Dissimilarity matrices of full alignments identified minimal divergence within BSN subspecies, but a ~2% dissimilarity across BSN subspecies (Table 12). Full mitochondrial sequences for most Texas BSN were 16,376 bp, with Hidalgo newts being

16,382 bp. Complete mitochondrial sequence was generated for a single Mexico sample and was 16,376 bp, equivalent to complete sequence size of all Texas samples other than Hidalgo County.

	MER1	MER2	MER3	MER4	MER5	KAL1	EU880322	EU880323	KP013091	EU880333	EU880334
MER1		6	7	7	9	48	18	157	206	380	372
MER2	0.156		7	7	9	50	18	159	208	383	374
MER3	0.182	0.182		4	8	49	15	158	206	381	373
MER4	0.182	0.182	0.104		8	49	15	159	207	382	372
MER5	0.234	0.234	0.208	0.208		50	19	160	206	378	370
KAL1	1.248	1.3	1.274	1.274	1.3		60	145	202	389	378
EU880322	0.461	0.461	0.383	0.383	0.487	1.553		169	217	379	383
EU880323	4.083	4.135	4.109	4.135	4.161	3.771	4.387		154	360	345
KP013091	5.358	5.41	5.358	5.384	5.358	5.254	5.635	4.005		389	376
EU880333	9.876	9.954	9.902	9.928	9.824	10.11	10	9.356	10.11		212
EU880334	9.675	9.727	9.701	9.675	9.623	9.831	9.951	8.973	9.779	5.507	

Table 14. Amino acid dissimilarity matrix between haplotypes identified in this study and GenBank accessions for *N. meridionalis* (EU880322), *N. viridescens* (EU880323), N. perstriatus (KP013091), *Taricha granulosa* (EU880333), and *T. rivulosa* (EU880334). Percent dissimilarity (below diagonal) and number of differences (above diagonal) are represented. Haplotype KAL2 not shown due to incomplete sequencing.

PartitionFinder identified 51 schemes for full sequence analysis and 20 schemes for partial sequence analysis. Selected full mitochondrial sequence scheme (Table 10) had an AICc value of 77784.7634304 and partial sequence scheme (Table 11) had an AICc value of 59774.8723408. Phylogenies from BA and MLA matched across full and partial mitochondrial sequence analyses (Figure 23 and Figure 24, respectively). Analyses with full mitochondrial DNA sequences (Figure 23) provided greater node support than partial sequence analyses (Figure 24) at the individual level (figure not shown), but both analyses resulted in the same topology. In all models, *N. perstriatus* and *N. viridescens* formed a monophyletic sister clade to *N. meridionalis*, with *Notophthalmus* forming a single monophyletic clade separate from *T. rivulose*. Described BSN subspecies were clearly distinct in all phylogenies. Genetic dissimilarity across concatenated regions studied by Whitmore et al. (2013) showed a 1.52% – 1.23% identity dissimilarity between BSN subspecies,

correlating to a roughly 1.88 – 1.52 mya (Figure 25) divergence between BSN subspecies following the 0.81% per million years identified by Whitmore et al. (2013).

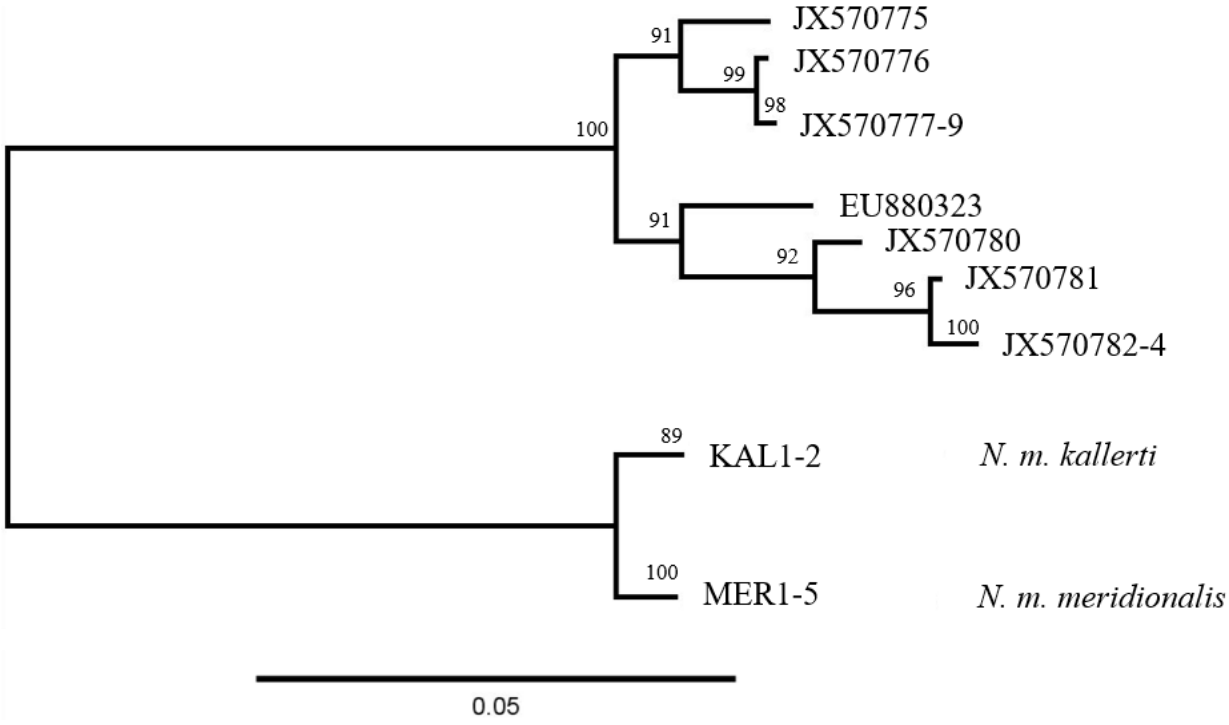

Figure 25. Phylogeny based on CytB, D-Loop, and tRNA-Pro genes used in Whitmore et al. (2013). Values presented are RAxML derived bootstrap values from Maximum Likelihood analysis based on 1000 bootstraps. Branches collapsed to represent patristic distances > 0.002.

Discussion

Allozyme data has suggested *N. meridionalis* and *N. perstriatus* forming a monophyletic clade, with *N. viridescens* as sister (Reilly, 1990), however incomplete mitochondrial genetic work has indicated *N. perstriatus* is sister to *N. viridescens* (Alexander Pyron & Wiens, 2011; Weisrock et al., 2006) with *N. meridionalis* being sister the rest other *Notophthalmus* species. Despite an update in 2008, the IUCN Red List does not recognize this discrepancy (IUCN). The full mitochondrial phylogeny generated by the present study supports placing *N. meridionalis* basal to the rest of *Notophthalmus* species.

To date there has been no genetic comparison between BSN subspecies. The literature is split regarding the appropriate taxonomic ranking for each subspecies, with some listing them as sister species (see review in Mecham, 1968b). Advances in genetics and sequencing technology have provided an opportunity for further investigation of the BSN species/subspecies debate, along with their contentious relation within *Notophthalmus* (see Weisrock et al., 2006 and Reilly 1990). Use of full mitochondrial sequences in this study suggests *kallerti* may be genetically distinctive with divergence just over 1.5 mya, supporting the subspecies delineation. Salamander mitochondria have a history of complexity seemingly unsuited for species level classification. Tiger salamanders (*Ambystoma*) have shown sequence divergence of over 4% within the D-loop structure in some subspecies (Shaffer & McKnight, 1996), while the ring-species *Ensatina eschscholtzii* can have up to 14% Cytb divergence across subspecies (Moritz, Schneider, & Wake, 1992). Even within the Central Newt clade, a ~4% divergence across Cytb and D-loop sequences was within a single subspecies between study sites within Iowa (Whitmore et al., 2013). Alternatively though, a 15% divergence across ND1, ND2, and COI sequences has been found to correlate to genera separation of Europe salamandrids (Weisrock, Macey, Ugurtas, Larson, & Papenfuss, 2001) and a 6.8% sequence divergence across ribosomal RNA and Cytb genes was sufficient for species delineation in *Salamandrina terdigitata* (Mattoccia, Romano, & Sbordoni, 2005). Within the BSN clade, full mitochondrial DNA sequences hardly diverged by 2%, unsupportive of species level separation.

Genetic studies on salamander phylogenies have relied on few BSN samples, all provided by Gladys Porter Zoo (Reilly, 1990; Weisrock et al., 2006; Zhang, Papenfuss, Wake, Qu, & Wake, 2008). This study provides a first look at genetic structure across wild BSN populations. Within the LRGV, newts show a high degree of relatedness (< 0.2% mitochondrial dissimilarity), but

variation is not correlated with geographic distance. Water management projects in the 1950's have greatly limited connectivity potential between resacas (Jahrsdoerfer & D. M. Leslie, 1988)). However, parts of the LRGV still flood after heavy rains providing prospects for continued connectivity and dispersal. Considering BSN behavior to seek refuge underground when ephemeral waters are dry (Bare & Kline, 2017), newts may not exhibit significant overland dispersal, as is common in the Eastern Newt (Regosin, Windmiller, Homan, & Reed, 2005; Roe & Grayson, 2008). Without greater overland dispersal, waterway connectivity would provide the best potential for gene flow among metapopulations.

Intriguingly, compared to Texas newts, Mexican newts have a closer identity to the Eastern Newt based on full mitochondrial sequence (Texas BSN ~9.15%, Mexico BSN ~8.95%) and amino acid analyses (157-160 differences compared to 145 differences and 25 shared AA consensus divergences compared to only 14), suggesting Texas BSN may have originated from a Mexican ancestor. This seemingly disjunct pattern of relatedness may be a result of glaciation events pushing the ancestral BSN range south into Mexico, separating it from other *Notophthalmus* species. Glaciation events are known to have significant impacts on species phylogenetics (G M Hewitt, 1999; Godfrey M Hewitt, 1996; Kuchta & Tan, 2005; Shafer, Cullingham, Côté, & Coltman, 2010; Starkey et al., 2003; Zinenko et al., 2015). For instance, a 4% sequence dissimilarity between *N. v. louisianensis* from north and south Iowa may be explained by glacial retreat followed by subsequent recolonization of glacial pools (Whitmore et al., 2013). Divergence date estimates provided by Whitmore et al. (2013) and Zhang et al. (2008), ~12 mya and 11.4 mya respectively, place *N. meridionalis* divergence from other *Notophthalmus* species during the mid-Miocene, a period of climate transition associated with increased evolutionary turnover (Flower &

Kennett, 1994). This assessment is based on limited sequences though and requires greater investigation.

This study was limited to LRGV samples, but attempted sampling farther north in Kenedy, Kleberg, and San Patricio counties. Efforts were also hampered by drought conditions in the LRGV during 2016 and 2017. In addition, of the eleven samples sequenced, one failed and two were only partial (both from Mexico). Nonetheless, this study offers a greater insight into BSN genetic structure than any before, providing support for the subspecies delineation with genetic evidence. Additional sequencing of nuclear genes would fortify the assessment made herein and provide a refined resolution to answer population connectivity questions. Future studies will benefit from additional sampling throughout the range of the species.

CHAPTER V

SUMMARY AND CONCLUSIONS

The Black-spotted Newt has suffered from a severe lack of study, both in Texas and Mexico. Of the little that is known, significant portions are contained in grey literature, unpublished reports, or are based on probable misidentification of Eastern newts (Mecham 1968b, Rappole & Klicka 1992, Judd 1985). Consequentially, the species is listed as globally endangered (IUCN, NatureServe) due to a lack of information rather than an assured threat. This purpose of this study was to amend some of these shortcomings and provide directions for further investigation.

High resolution habitat suitability maps were generated during this study and offer probability estimates based on historical data and environmental variables. These maps should aid to focus future investigations, significantly reducing search effort in regions unlikely to have black-spotted newts in favor of high likelihood areas; historical records should still provide further guidance though. Maps provided herein are based on subsets of records that had sufficient geographic location data and therefore may exclude regions that were not accurately listed in records. As more locales are catalogued, these maps will need to be updated. In addition, climate change distribution models will be indispensable for predicting BSN range shifts and population viability (Hijmans & Graham, 2006; Milanovich, Peterman, Nibbelink, & Maerz, 2010). How climate change will impact the species is currently unknown, but there is potential for range reductions or shifts in the near future (Milanovich et al., 2010). Such areas should be a focus for

conservation efforts. Previous assessments have assumed land use to influence BSN habitat suitability (Judd, 1985; Rappole & Klicka, 1991), as it is a major factor in amphibian declines (Brum et al., 2013; Gibbons et al., 2006; Steen & Gibbs, 2005). Models generated for this study did not incorporate land use factors, in part because that was not the aim of this study, but also because current understanding of land use impact is contentious considering that the species has been documented in a variety of persistent heavily modified locations (Thornton, 1977; this study, see Chapter III).

Black-spotted Newt genetics has been even less explored in the literature than distribution, with only three articles recognized (K. H Kozak, Weisrock, & Larson, 2006; Reilly, 1990; Zhang & Wake, 2009) to have used *N. meridionalis.* Despite modern genetic analyses (Weisrock et al., 2006; Zhang, Papenfuss, Wake, Qu, & Wake, 2008), allozyme relationships (Reilly, 1990) have continued to be cited by prominent conservation groups, such as the IUCN and AmphibiaWeb. This study provided the most genetic data on *N. meridionalis* to date, including the first genetic records for the southern subspecies, *N. m. kallerti*. Phylogenetic analyses conducted herein provided consensus with genetic lineages identified by Weisrock et al. (2006) and Zhang & Wake (2008), recognizing *N. meridionalis* to be the most basal lineage of the *Notophthalmus* genus with clear genetic separation of the northern and southern forms in agreeance with the morphometric determination (Mecham, 1968b). Furthermore, molecular clock estimates based on work with *N. viridescens* (Whitmore, Losee, Meyer, & Spradling, 2013) provides the first estimate of divergence between the two forms to be between 1.5 and 1.8 mya. When comparing nucleotide and amino acid divergences, it was also discovered that the southern subspecies (*N. m. kallerti*) has a greater similarity to the sister species *N. viridescens* than does the northern subspecies (*N. m.*

meridionalis). Confirmation of this relationship requires further study, but does suggest that sympatry in San Patricio County (Mecham, 1968a) is an evolutionarily recent occurrence.

This study was primarily focused on the Lower Rio Grande Valley (LRGV) and future work will provide opportunity to collect greater inter- and intra-population genetic data from across the range. However, even in historically active sites newts are highly cryptic, making genetic collections an arduous task. After two years of investigation, Judd (1985) was only able to document three populations. Similarly, Rappole and Klicka (1991) designated nine metapopulation regions after three years of study. With such low returns, different field tactics are needed to survey for black-spotted newts. This study worked on developing two techniques - one for aquatic surveying and one for terrestrial surveying - to improve future surveying efforts and investigated relative effectiveness of more traditional methods.

Environmental DNA (eDNA) was investigated in this study to ascertain its effectiveness. Due to high turbidity levels in local pond waters, many traditionally used filter sizes would clog, drastically reducing the potential amount of DNA collected. As an alternative, this study found that use of a hand press and filters intended for coffee were suitable replacements for the bulkier and more expensive materials used for eDNA. While traditional filters were able to collect sufficient BSN DNA from 50 mL of spiked trials, field concentrations of eDNA can be much lower. The additional use of nested primers developed in this study allow substantial amplification for low concentration extracts and are BSN specific.

For terrestrial investigations, the use of borescope technology and placement of artificial burrows provides promise and may offer a novel survey method for this elusive species. It should be noted that due to extensive private ownership in the RGV, use of artificial burrows may prove difficult to implement on a wide-scale. Furthermore, burrow installation can be labor intensive.

There is also risk that native spiders commandeering burrows as refuge, preventing newt habitation. Comparatively though, artificial burrows offer a passive trap in place of pitfall traps which are more labor intensive, require frequent visitation to reduce mortality risk, and have not been successful for BSN sampling (Rappole & Klicka, 1991; this study) .Future studies should look to incorporate such methods to broaden investigative scope and increase detection and sampling rates. More samples will improve future genetic analyses and sensitive detection capabilities will improve occurrence data for distribution analyses.

During this study, ten BSN sites across eight localities (two sites from both Palo Alto National Battlefield and Laguna Atascosa National Wildlife Refuge) were found, from which over 40 tissue samples were collected. Six sites were previously undocumented, four of which were publicly owned. Historical BSN sites within the study area were also investigated (Figure 14), but newts were not found. It is necessary to report that even if newts were not located in these and other sites, it does not mean they are not present as this species displays highly cryptic behavior. Additionally, vast stretches of ranchland throughout Kenedy and Kleberg counties were inaccessible for this study but were estimated to be suitable habitat based on MaxEnt modeling (see Chapter I). Cattle ponds (Judd, 1985; Rappole & Klicka, 1991) and agricultural sites (Thornton 1977) have had black-spotted newts with similar findings during this study, seemingly counter to presumptions.

Within the LRGV, more specifically Cameron County, the species is frequently found in resaca systems and oxbow lakes (Judd, 1985; Kazmaier, 2009; Oliver, Chaney, Miller, & Parker, 1980; Rappole & Klicka, 1991). During this study, five of seven BSN sites in Cameron County were known to be resacas or oxbows as well, two of which were also historically cattle troughs. Due to close association with resaca systems that historical interconnected during floods, black-

spotted newts may experience distinct fine-scaled resaca lineages similar to those found in stream-associated *Eurycea* (Kenneth H. Kozak, Blaine, & Larson, 2006). To take advantage of BSN wetland association, conservation efforts should advise ranchlands to install cattle dugouts within historic wetlands or resaca systems for greater BSN habitat availability and ease for colonization.

The paltry amount of research on the Black-spotted Newt is concerning. As a Globally Endangered species (IUCN), it is assumed that populations are threatened and in decline due to a consortium of factors. However, no significant research has been done to confirm any such impacts. In fact, it is likely that this species is more common than originally perceived, with population pockets scattered within privately owned ranches closed to biological study (Scott, 1996). With the aid of distribution maps and surveying techniques described during this study, BSN monitoring should become easier. As more populations are found, data can be added to the distribution modeling procedure and to the genetic database. With the incorporation of genetic data, distribution models can further specify areas of consideration for surveys. In this fashion, the work conducted for this study provides a feed-back mechanism to generate more refined models and accelerate data collection on this threatened species.

Conservation Status

The NatureServe organization provides comprehensive reviews and conservation statuses of species that are frequently cited by scientists and conservation groups. *Notophthalmus meridionalis* is listed as have an S2 ranking for the state of Texas (NatureServe, 2016), in line with the Threatened status in the state (Texas Parks and Wildlife Department, 2000). Following from the advancement of knowledge regarding this species in Texas, a recalculation of this status was conducted the Rank Estimator Calculator (v3.18) form from NatureServe. Data from this study and a comprehensive literature review was used to estimate impact values, population numbers,

and threat assessments. The Calculated Rank was S2S3, with a final assigned rank of S3. A Global rank estimation was not considered as this study did not conducted research in Mexico.

The Black-spotted Newt has been petitioned for listing on the Endangered Species Act (Rosmarino & Tutchton, 2007), and is up for review in the coming years. An S3 rank was assigned based on current understanding of the species, but also takes into consideration the difficulties that may be encountered if the species is federally listed. Over the course of this study, property access was requested of private landowners but was frequently denied due to a pervasive concern that property rights may be taken away if the species was discovered. If this newt were to be listed, research may become inadvertently restricted to public lands, greatly reducing the scope of research. One of the greatest threats to this species is a lack of knowledge, primarily regarding population status and trends. During the last three major studies on this species (Judd, 1985; Rappole & Klicka, 1991; this study), new localities continued to be documented, suggesting a substantial number of populations persist and have yet to be discovered. By maintaining a Threatened status, future investigations will be less hampered by private citizen concerns and will be able to gather data at a greater pace.

REFERENCES

Alexander Pyron, R. and John J. Wiens. 2011. "A Large-Scale Phylogeny of Amphibia Including over 2800 Species, and a Revised Classification of Extant Frogs, Salamanders, and Caecilians." *Molecular Phylogenetics and Evolution* 61(2):543–83. Retrieved (http://dx.doi.org/10.1016/j.ympev.2011.06.012).

Alexandrino, J., E. Froufe, J. W. Arntzen, and N. Ferrand. 2000. "Genetic Subdivision, Glacial Refugia and Postglacial Recolonization in the Golden-Striped Salamander, *Chioglossa lusitanica* (Amphibia: Urodela)." *Molecular Ecology* 9(6):771–81.

Allen, Joel Asaph. 1876. *American Bisons, Living and Extinct*. (Vol. 10). University Press, Welch, Bigelow.

Andersen, Mark and Gary Beauvais. 2013. *Predictive Distribution Modeling of Species of Greatest Conservation Need in Texas*. Austin, Texas.

Anderson, Robert P. and Ali Raza. 2010. "The Effect of the Extent of the Study Region on GIS Models of Species Geographic Distributions and Estimates of Niche Evolution: Preliminary Tests with Montane Rodents (Genus Nephelomys) in Venezuela." *Journal of Biogeography* 37(7):1378–93.

Aurelio, Ramírez Bautista, Fernando Mendoza-Quijano, Hernandez Ibarra Xochitl, and Tovar Tovar Hector. 2004. "Información General Información Taxonómica Distribución." 1–5.

Babb, Randall D. et al. 2015. *Amphibians and Reptiles of the US-Mexico Border States*. 1st ed. edited by J. A. Lemos-Espinal. College Station, TX: Texas A&M University Press.

Bare, Evan and Richard Kline. 2017. "Habitat Use - *Notophthalmus meridionalis* (Black-Spotted Newt)." *Herpetological Review* 48(4):827–28.

Beebee, T. J. C. 2005. "Conservation Genetics of Amphibians." *Heredity* 95(6):423–27. Retrieved (http://www.nature.com/doifinder/10.1038/sj.hdy.6800736).

Best, Michael L. and Hartwell H. Welsh, Jr. 2014. "The Trophic Role of a Forest Salamander: Impacts on Invertebrates, Leaf Litter Retention, and the Humification Process." *Ecosphere* 5(2):art16. Retrieved (http://doi.wiley.com/10.1890/ES13-00302.1).

Biggs, Jeremy et al. 2015. "Using eDNA to Develop a National Citizen Science-Based Monitoring Programme for the Great Crested Newt (*Triturus cristatus*)." *Biological Conservation* 183:19–28. Retrieved (http://dx.doi.org/10.1016/j.biocon.2014.11.029).

Bishop, Sherman C. 1947. *Handbook of Salamanders: The Salamanders of the United States, of Canada, and of Lower California*. Ithaca, NY: Comstock Publishing Company, Inc.

Bista, Iliana et al. 2017. "Annual Time-Series Analysis of Aqueous eDNA Reveals Ecologically Relevant Dynamics of Lake Ecosystem Biodiversity." *Nature Communications* 8:14087. Retrieved (http://www.nature.com/doifinder/10.1038/ncomms14087).

Bodinof Jachowski, Catherine M., Joshua J. Millspaugh, and William A. Hopkins. 2016. "Current Land Use Is a Poor Predictor of Hellbender Occurrence: Why Assumptions Matter When Predicting Distributions of Data-Deficient Species." *Diversity and Distributions* 22(8):865–80.

Boulenger, G. A. 1888. "On a Rare American Newt, *Molge meridionalis*, Cope." *The Annals and Magazine of Natural History* 1(6):24.

Boundy, Jeff. 1994. "*Notophthalmus meridionalis*." *Herpetological Review* 25(3):129. Retrieved (http://www.jstor.org/stable/1930594?origin=crossref).

Brame, A. H.Jr. 1972. "A Checklist of the Living and Fossil Salamanders (Order Caudata) of the World."

Brandon, Ronald A. 1965. "Morphological Variation and Ecology of the Salamander *Pheognathus hubrichti*." *Copeia* 1965(1):67–71.

Brum, Fernanda Thiesen et al. 2013. "Land Use Explains the Distribution of Threatened New World Amphibians Better than Climate." *PLoS ONE* 8(4):4–11.

Bsal Task Force. 2016. *Bsal Task Force: 2016 Annual Report*. Retrieved (http://investors.whirlpoolcorp.com/common/download/download.cfm?companyid=ABEA-5DXEK8&fileid=931320&filekey=110957C3-5A45-41DF-87D2-34714A8AA1B7&filename=Whirlpool_2016_Annual_Report.pdf).

Burton, Thomas M. and Gene E. Likens. 1975. "Energy Flow and Nutrient Cycling in Salamander Populations in the Hubbard Brook Experimental Forest, New Hampshire." *Ecology* 56(5):1068–80.

Burton, Thomas M. and Gene E. Likens. 1975. "Salamander Populations and Biomass in the Hubbard Brook Experimental Forest , New Hampshire." *Copeia* 1975(3):541–46.

Van Buskirk, J. and B. R. Schmidt. 2000. "Predator-Induced Phenotypic Plasticity in Larval Newts: Trade-Offs, Selection, and Variation in Nature." *Ecology* 81(11):3009–28.

Butler, Joseph A. and Alan Harris. 2010. "Determining Gopher Tortoise Burrow Occupancy Using a Robotic Camera." 1–21.

Cai, Peng, Qiao Y. Huang, and Xue W. Zhang. 2006. "Interactions of DNA with Clay Minerals and Soil Colloidal Particles and Protection against Degradation by DNase." *Environmental Science and Technology* 40(9):2971–76.

Canals, R. M., V. Ferrer, L. San Emeterio, A. Iritarte, and E. Villanueva. 2010. “Water Quality and the Environmental Use of Livestock Ponds.” *Grassland Science in Europe* 15:1037–39. Retrieved (http://www.cabdirect.org/abstracts/20103325745.html).

Canestrelli, Daniele, Florinda Sacco, and Giuseppe Nascetti. 2012. “On Glacial Refugia, Genetic Diversity, and Microevolutionary Processes: Deep Phylogeographical Structure in the Endemic Newt *Lissotriton italicus*.” *Biological Journal of the Linnean Society* 105(1):42–55.

Carbajal-Marquez, Ruben Alonso, Gustavo E. Quintero-Diaz, and Guillermo Martinez De la Vega. 2014. “Geographic Distribution -*Notophthalmus meridionalis* (Black-Spotted Newt).” *Herpetological Review* 45(4l):650.

Chambers, William T. 1930. “Lower Rio Grande Valley of Texas.” *Economic Geography* 6(4):364–73.

Chen, I. C., J. K. Hill, R. Ohlemuller, D. B. Roy, and C. D. Thomas. 2011. “Rapid Range Shifts of Species Associated with High Levels of Climate Warming.” *Science* 333(6045):1024–26. Retrieved (http://www.sciencemag.org/cgi/doi/10.1126/science.1206432).

Chetan, Nag, Karanth K. Praveen, and Gururaja Kotambylu Vasudeva. 2014. “Delineating Ecological Boundaries of Hanuman Langur Species Complex in Peninsular India Using MaxEnt Modeling Approach.” *PLoS ONE* 9(2):1–11.

Church, Sheri A., Johanna M. Kraus, Joseph C. Mitchell, Don R. Church, and Douglas R. Taylor. 2003. “Evidence for Multiple Pleistocene Refugia in the Postglacial Expansion of the Eastern Tiger Salamander, *Ambystoma tigrinum tigrinum*.” *Evolution* 57(2):372–83.

Clover, Elzada U. 1937. “Vegetational Survey of the Lower Rio Grande Valley, Texas.” *California Botanical Society* 4(2):41–66.

Conant, Roger and Joseph T. Collins. 1998. *Reptiles and Amphibians: Eastern and Central North America*. 3rd ed. edited by R. T. Peterson. Houghton Mifflin Company.

Cope, ED. 1880. “On the Zoological Position of Texas.” *Bulletin of the U. S. National Museum* 17:1–51.

Cope, ED. 1889. “The Batrachia of North America.” *Bulletin of the U. S. National Museum* 34:5–525.

Cosentino, Bradley J. et al. 2014. “Citizen Science Reveals Widespread Negative Effects of Roads on Amphibian Distributions.” *Biological Conservation* 180:31–38. Retrieved (http://dx.doi.org/10.1016/j.biocon.2014.09.027).

Crother, Brian I. 2012. *Scientific and Standard English Names of Amphibians and Reptiles of North America North of Mexico, with Comments Regarding Confidence in Our Understanding*. Retrieved (http://www.ssarherps.org).

Cushman, Samuel A. 2006. “Effects of Habitat Loss and Fragmentation on Amphibians: A Review and Prospectus.” *Biological Conservation* 128(2):231–40.

Davic, Robert D. and Hartwell H. Welsh. 2004. “On the Ecological Roles of Salamanders.” *Annual Review of Ecology, Evolution, and Systematics* 35(1):405–34. Retrieved (http://www.annualreviews.org/doi/10.1146/annurev.ecolsys.35.112202.130116).

Davis, M. B. 2001. “Range Shifts and Adaptive Responses to Quaternary Climate Change.” *Science* 292(5517):673–79. Retrieved (http://www.sciencemag.org/cgi/doi/10.1126/science.292.5517.673).

Dejean, Tony et al. 2012. “Improved Detection of an Alien Invasive Species through Environmental DNA Barcoding: The Example of the American Bullfrog *Lithobates catesbeianus*.” *Journal of Applied Ecology* 49(4):953–59.

Díaz-Rodríguez, Jesús et al. 2015. “Molecular Evidence for Cryptic Candidate Species in Iberian Pelodytes (Anura, Pelodytidae).” *Molecular Phylogenetics and Evolution* 83:224–41.

Doi, Hideyuki et al. 2015. “Use of Droplet Digital PCR for Estimation of Fish Abundance and Biomass in Environmental DNA Surveys.” *PLoS ONE* 10(3):1–11.

Dunker, KJ Kristine J. et al. 2016. “Potential of Environmental DNA to Evaluate Northern Pike (*Esox lucius*) Eradication Efforts : An Experimental Test and Case Study.” *PLoS ONE* 11(9):1–21. Retrieved (http://dx.plos.org/10.1371/journal.pone.0162277).

Dunn, E. R. 1918. “The Collection of Amphibia Caudata of the Museum of Comparative Zoology.” *Bulletin of the Museum of Comparative Zoology* 62(9):445–71.

Eckert, C. G., K. E. Samis, and S. C. Lougheed. 2008. “Genetic Variation across Species’ Geographical Ranges: The Central-Marginal Hypothesis and beyond.” *Molecular Ecology* 17(5):1170–88.

Elith, Jane et al. 2006. “Novel Methods Improve Prediction of Species’ Distributions from Occurrence Data.” *Ecography* 29(2):129–51.

Evans, Nathan T. et al. 2016. “Quantification of Mesocosm Fish and Amphibian Species Diversity via Environmental DNA Metabarcoding.” *Molecular Ecology Resources* 16(1):29–41.

Fauth, John E., William J. Resetarits, and William J. Resetarits Jr.. 1991. “Interactions Between the Salamander *Siren intermedia* and the Keystone Predator *Notophthalmus viridescens*.” *Ecology* 72(3):827–38.

Feldmeier, Stephan et al. 2016. “Exploring the Distribution of the Spreading Lethal Salamander Chytrid Fungus in Its Invasive Range in Europe - A Macroecological Approach.” *PLoS ONE* 11(10):1–17.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. “Species Detection Using

Environmental DNA from Water Samples." *Biology Letters* 4(4):423–25. Retrieved (http://rsbl.royalsocietypublishing.org/cgi/doi/10.1098/rsbl.2008.0118).

Ficetola, Gentile Francesco, Carlo Rondinini, Anna Bonardi, Daniele Baisero, and Emilio Padoa-Schioppa. 2015. "Habitat Availability for Amphibians and Extinction Threat: A Global Analysis." *Diversity and Distributions* 21(3):302–11.

Fick, S. E. and R. J. Hijmans. 2017. "Worldclim 2: New 1-Km Spatial Resolution Climate Surfaces for Global Land Areas." *International Journal of Climatology*.

Flores-Villela, Oscar, Gabriela Parra-Olea, G. A. Hemmerson, D. Wake, and K. Irwin. 2008. "*Notophthlamus meridionalis*." *The IUCN Red List of Threatened Species 2008*. Retrieved October 2, 2017 (http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T59452A11944420.en).

Flores, Sandra Denisse Herrera. 2010. *NORMA Oficial Mexicana NOM-059-SEMARNAT-2010, Protección Ambiental-Especies Nativas de México de Flora Y Fauna Silvestres-Categorías de Riesgo Y Especificaciones Para Su Inclusión, Exclusión O Cambio-Lista de Especies En Riesgo*.

Frank, Glaw and Jorn Kohler. 1998. "Amphibian Species Diversity Exceeds that of Mammals." *Herpetological Review* 29(1):11–12.

Freda, Joseph. 1991. "The Effects of Aluminum and Other Metals on Amphibians." *Environmental Pollution* 71(2–4):305–28.

Frías-Alvarez, Patricia, J.Jaime Zúñiga-Vega, and Oscar Flores-Villela. 2010. "A General Assessment of the Conservation Status and Decline Trends of Mexican Amphibians." *Biodiversity and Conservation* 19(13):3699–3742.

Frisbie, M. P. and R. L. Wyman. 1992. "The Effect of Environmental pH on Sodium Balance in the Red-Spotted Newt, *Notophthalmus viridescens*." *Arch Environ Contam Toxicol* 23(192344439):64–68.

Frisbie, Malcolm Pratt and Richard L. Wyman. 1991. "The Effects of Soil pH on Sodium Balance in the Red-Backed Salamander, *Plethodon cinereus*, and Three Other Terrestrial Salamanders." *Physiological Zoology* 64(4):1050–68. Retrieved (http://www.jstor.org/stable/30157956).

Frost, Darrel R. 2017. "Amphibian Species of the World: An Online Reference. Version 6.0." *American Museum of Natural History, New York, USA*. Retrieved October 3, 2017 (http://research.amnh.org/herpetology/amphibia/index.html).

Funk, W.Chris, Marcel Caminer, and Santiago R. Ron. 2012. "High Levels of Cryptic Species Diversity Uncovered in Amazonian Frogs." *Proceedings of the Royal Society B* 279:1806–14.

Gamble, Lloyd, Sai Ravela, and Kevin McGarigal. 2008. "Multi-Scale Features for Identifying Individuals in Large Biological Databases: An Application of Pattern Recognition

Technology to the Marbled Salamander, *Ambystoma opacum*." *Journal of Applied Ecology* 45(1):170–80.

Garcia, G. A., Fernando Mendoza-Quijano, and Sol de Mayo Mejenes-Lopez. 2003. "A Range of Extension for *Notophthalmus meridionalis kallerti* Wolterstorff (Caudata: Salamandridae) in Veracruz, México." *Acta Vertebrata* 25–27.

Gibbons, J.Whitfield et al. 2006. "Remarkable Amphibian Biomass and Abundance in an Isolated Wetland: Implications for Wetland Conservation." *Conservation Biology* 20(5):1457–65.

Gill, Douglas E. 1978. "The Metapopulation Ecology of the Red-Spotted Newt, *Notophthalmus viridescens* (Rafinesque)." *Ecological Monographs* 48(2):145–66.

Gill, Douglas E. 1979. "Density Dependence and Homing Behavior in Adult Red-Spotted Newts, *Notophthalmus viridescens* (Rafinesque)." *Ecology* 60(4):800–813.

Giovannini, Andrea, Daniele Seglie, and Cristina Giacoma. 2014. "Identifying Priority Areas for Conservation of Spadefoot Toad, *Pelobates fuscus insubricus*, Using a Maximum Entropy Approach." *Biodiversity and Conservation* 23(6):1427–39.

Glista, David J., Travis L. DeVault, and J.Andrew DeWoody. 2008. "Vertebrate Road Mortality Predominantly Impacts Amphibians." *Herpetological Conservation and Biology* 3(1):77–87.

Goldberg, Caren S., David S. Pilliod, Robert S. Arkle, and Lisette P. Waits. 2011. "Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders." *PLoS ONE* 6(7).

Gorički, Špela et al. 2017. "Environmental DNA in Subterranean Biology: Range Extension and Taxonomic Implications for Proteus." *Scientific Reports* 7(October 2016):45054. Retrieved (http://www.nature.com/articles/srep45054).

Gray, Matthew J. et al. 2015. "Batrachochytrium Salamandrivorans: The North American Response and a Call for Action." *PLoS Pathogens* 11(12):1–9.

Grayson, Kristine L. and Andrew W. Roe. 2007. "Glow Sticks as Effective Bait for Capturing Aquatic Amphibians in Funnel Traps." *Herpetological Review* 38(2):168–70.

Greenwald, Katherine R., H.Lisle Gibbs, and Thomas A. Waite. 2009. "Efficacy of Land-Cover Models in Predicting Isolation of Marbled Salamander Populations in a Fragmented Landscape." *Conservation Biology* 23(5):1232–41.

Greenwald, Katherine R., Jennifer L. Purrenhage, and Wesley K. Savage. 2009. "Landcover Predicts Isolation in Ambystoma Salamanders across Region and Species." *Biological Conservation* 142(11):2493–2500. Retrieved (http://dx.doi.org/10.1016/j.biocon.2009.05.021).

Groff, Luke A., Sharyn B. Marks, and Marc P. Hayes. 2014. "Using Ecological Niche Models to Direct Rare Amphibian Surveys: A Case Study Using the Oregon Spotted Frog (*Rana pretiosa*)." *Herpetological Conservation and Biology* 9(2):354–68.

Grosjean, Stéphane, Annemarie Ohler, Yodchaiy Chuaynkern, Corinne Cruaud, and Alexandre Hassanin. 2015. "Improving Biodiversity Assessment of Anuran Amphibians Using DNA Barcoding of Tadpoles. Case Studies from Southeast Asia." *Comptes Rendus - Biologies* 338:351–361.

Group, Ecoclub Amphibian et al. 2016. "Citizen Scientists Monitor a Deadly Fungus Threatening Amphibian Communities in Northern Coastal California, Usa." *Journal of Wildlife Diseases* 52(3):516–23. Retrieved (http://www.jwildlifedis.org/doi/10.7589/2015-10-280).

Guardiola, Magdalena et al. 2016. "Spatio-Temporal Monitoring of Deep-Sea Communities Using Metabarcoding of Sediment DNA and RNA." *PeerJ* 4:e2807. Retrieved (https://peerj.com/articles/2807).

Ha, Hoehun and Fraser Shilling. 2017. "Modelling Potential Wildlife-Vehicle Collisions (WVC) Locations Using Environmental Factors and Human Population Density: A Case-Study from 3 State Highways in Central California." *Ecological Informatics* (October). Retrieved (http://linkinghub.elsevier.com/retrieve/pii/S1574954117302157).

Hamilton, Wayne T. 2003. "Drought: Managing for and During the Bad Years." Pp. 133–52 in *Ranch Management: Integrating Cattle, Wildlife, and Range*, edited by C. A. Forgason, F. C. Bryant, and P. C. Genho. King Ranch, Kingsville, TX.

Hanken, James. 1999. "Why Are There so Many New Amphibian Species When Amphibians Are Declining?" *Trends in Ecology & Evolution* 14(1):7–8. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/10234239).

Harwood, Paul D. 1930. "A New Species of Oxysomatium (Nematoda) with Some Remarks on the Genera Oxysomatium and Aplectana, and Observations on the Life History." *The Journal of Parasitology* 17(2):61–73.

Harwood, Paul D. 1932. "The Helminths Parasitic in the Amphibia and Reptilia of Houston, Texas, and Vicinity." *Proceedings of the United States National Museum* 81(2940):1–71.

Hengl, T. et al. 2017. "SoilGrids250m: Global Gridded Soil Information Based on Machine Learning." *PLoS ONE* 12(2):e0169748.

Hernandez, Pilar A., Catherine Graham, Lawrence L. Master, and Deborah L. Albert. 2006. "The Effect of Sample Size and Species Characteristics on Performance of Different Species Distribution Modeling Methods." *Ecography* 29(5):773–85. Retrieved (http://dx.doi.org/10.1111/j.0906-7590.2006.04700.x%5Cnhttp://onlinelibrary.wiley.com/store/10.1111/j.0906-7590.2006.04700.x/asset/j.0906-7590.2006.04700.x.pdf?v=1&t=hrlipes8&s=e73a0337239ec73c1461a1a6b7f68a0a576bf4c

7).

Herre, Wolf. 1936. “Ueber Rasse Und Artbildung.” *Abhandlungen Und Berichte Aus Dem Museum Für Natur- Und Heimatkunde Zu Magdeburg* 6(3):193–221.

Hewitt, G. M. 1999. “Postglacial Re-Colonisation of European Biota.” *Biological Journal of the Linnean Society* 68:87–112.

Hewitt, Godfrey M. 1996. “Some Genetic Consequences of Ice Ages, and Their Role in Divergence and Speciation.” *Biological Journal of the Linnean Society* 58(February):247–76.

Hibbs, Barry J. and Radu Boghici. 1999. “On the Rio Grande Aquifer: Flow Relationships, Salinization, and Environmental Problems From El Paso to Fort Quitman, Texas.” *Environmental & Engineering Geoscience* V(1):51–59.

Hicks, Norman G. and Scott M. Pearson. 2003. “Salamander Diversity and Abundance in Forests with Alternative Land Use Histories in the Southern Blue Ridge Mountains.” *Forest Ecology and Management* 177:117–30.

Highton, Richard and T.Preston Webster. 1976. “Geographic Protein Variation and Divergence in Populations of the Salamander *Plethodon cinereus*.” *Evolution* 30(March):33–45.

Hijmans, Robert J. and Catherine H. Graham. 2006. “The Ability of Climate Envelope Models to Predict the Effect of Climate Change on Species Distributions.” *Global Change Biology* 12(12):2272–81.

Hof, Christian, Miguel B. Araújo, Walter Jetz, and Carsten Rahbek. 2011. “Additive Threats from Pathogens, Climate and Land-Use Change for Global Amphibian Diversity.” *Nature* 480(7378):516–19.

Holman, Alan and Alisa J. Winkler. 1987. *A Mid-Pleistocene (Irvingtonian) Herpetofauna From a Cave in Southcentral Texas*. Austin, Texas.

Hoverman, Jason T., Matthew J. Gray, Debra L. Miller, and Nathan A. Haislip. 2012. “Widespread Occurrence of Ranavirus in Pond-Breeding Amphibian Populations.” *EcoHealth* 9(1):36–48.

Irwin, K. J. 1993. *A Preliminary Survey, with Management Recommendations on the Herpetofauna of the Lower Rio Grande Valley National Wildlife Refuge*. US Fish & Wildlife Service. Final Report. Alamo, Texas.

IUCN. 2015. *The IUCN Red List of Threatened Species* ™.

Jackson, Craig R. and Mark P. Robertson. 2010. “Predicting the Potential Distribution of an Endangered Cryptic Subterranean Mammal from Few Occurrence Records.” *Journal for Nature Conservation*. Retrieved (http://dx.doi.org/10.1016/j.jnc.2010.06.006).

Jackson, Craig R. and Mark P. Robertson. 2011. "Predicting the Potential Distribution of an Endangered Cryptic Subterranean Mammal from Few Occurrence Records." *Journal for Nature Conservation* 19(2):87–94.

Jahrsdoerfer, S. E. and D. M.Jr. Leslie. 1988. "Tamaulipan Brushland of the Lower Rio Grande Valley of South Texas: Description, Human Impact, and Management Options." *Biological Report* 88(36):63.

Johansson, Markus, Craig R. Primmer, and Juha Merilä. 2007. "Does Habitat Fragmentation Reduce Fitness and Adaptability? A Case Study of the Common Frog (*Rana temporaria*)." *Molecular Ecology* 16(13):2693–2700.

Joly, Pierre, Claire Morand, and Aurélie Cohas. 2003. "Habitat Fragmentation and Amphibian Conservation: Building a Tool for Assessing Landscape Matrix Connectivity." *Comptes Rendus Biologies* 326:132–39. Retrieved (http://linkinghub.elsevier.com/retrieve/pii/S1631069103000507).

Judd, Frank. W. 1985. *Status of Siren Intermedia Texana, Notophthlamus Meridionalis, and Crotaphytus Reticulatus*. Washington, D.C.

Kazmaier, Richard. 2009. *Summary of Activities Conducted at Laguna Atascosa NWR in 2005-2009 by West Texas A&M University*.

Klymus, Katy E., Catherine A. Richter, Duane C. Chapman, and Craig Paukert. 2015. "Quantification of eDNA Shedding Rates from Invasive Bighead Carp, *Hypophthalmichthys nobilis*, and Silver Carp, *Hypophthalmichthys molitrix*." *Biological Conservation* 183:77–84.

Köhler, Jörn et al. 2005. "New Amphibians and Global Conservation: A Boost in Species Discoveries in a Highly Endangered Vertebrate Group." *BioScience* 55(8):693–96.

Kolozsvary, Mary B. and Robert K. Swihart. 1999. "Habitat Fragmentation and the Distribution of Amphibians: Patch and Landscape Correlates in Farmland." *Canadian Journal of Zoology* 77(8):1288–99.

Kozak, Kenneth H., Russell A. Blaine, and Allan Larson. 2006. "Gene Lineages and Eastern North American Palaeodrainage Basins: Phylogeography and Speciation in Salamanders of the *Eurycea bislineata* Species Complex." *Molecular Ecology* 15(1):191–207.

Kuchta, Shawn R. and A. N.Min. Tan. 2005. "Isolation by Distance and Post-Glacial Range Expansion in the Rough-Skinned Newt, *Taricha granulosa*." *Molecular Ecology* 14(1):225–44.

Kumar, Sunil and Thomas J. Stohlgren. 2009. "Maxent Modeling for Predicting Suitable Habitat for Threatened and Endangered Tree *Canacomyrica monticola* in New Caledonia." *Journal of Ecology and Natural Science* 1(4):094–098. Retrieved (http://warnercnr.colostate.edu/~sunil/Kumar and Stohlgren_2009_endangered_tree_habitat_New_Caledonia.pdf).

LaFortune, Taylor Courtney. 2015. “Species Identification and Habitat Assessment of the South Texas Siren.” University of Texas Brownsville.

Laking, Alexandra E., Hai Ngoc Ngo, Frank Pasmans, An Martel, and Tao Thien Nguyen. 2017. “Batrachochytrium Salamandrivorans Is the Predominant Chytrid Fungus in Vietnamese Salamanders.” *Scientific Reports* 7 (October 2016):44443. Retrieved (http://www.nature.com/articles/srep44443).

Langen, Tom A., Kimberly M. Ogden, and Lindsay L. Schwarting. 2009. “Predicting Hot Spots of Herpetofauna Road Mortality Along Highway Networks.” *Journal of Wildlife Management* 73(1):104–14. Retrieved (http://www.bioone.org/doi/abs/10.2193/2008-017).

Leslie Jr., David M. 2016. *An International Borderland of Concern : Conservation of Biodiversity in the Lower Rio Grande Valley Scientific Investigations Report 2016 – 5078*. Reston, Virginia.

Liner, E. A. and G. Casas-Andreu. 2008. *Standard Spanish, English and Scientific Names of the Amphibians and Reptiles of Mexico*. Herpetolog.

Loiselle, Bette A. et al. 2008. “Predicting Species Distributions from Herbarium Collections: Does Climate Bias in Collection Sampling Influence Model Outcomes?” *Journal of Biogeography* 35(1):105–16.

Lozier, J. D., P. Aniello, and M. J. Hickerson. 2009. “Predicting the Distribution of Sasquatch in Western North America: Anything Goes with Ecological Niche Modelling.” *Journal of Biogeography* 36(9):1623–27.

MacDonald, Anna J. and Stephen D. Sarre. 2017. “A Framework for Developing and Validating Taxon-Specific Primers for Specimen Identification from Environmental DNA.” *Molecular Ecology Resources* 17(4):708–20.

Malstrom, Howard L. and Wayne R. Jordan. 1994. *Environmental Issues of the U.S.-Mexico Border Region: A Workshop Summary*. Retrieved (http://hdl.handle.net/1969.1/6166).

Martin, Paul S., C. R. Robins, and W. B. Heed. 1954. “Birds and Biogeography of the Sierra de Tamaulipas, an Isolated Pine-Oak Habitat.” *The Wilson Bulletin* 66(1):38–57.

Matheson, Carney D., Carli Gurney, Neal Esau, and Ryan Lehto. 2010. “Assessing PCR Inhibition from Humic Substances.” *The Open Enzyme Inhibition Journal* 3:38–45.

Mattoccia, Marco, Antonio Romano, and Valerio Sbordoni. 2005. “Mitochondrial DNA Sequence Analysis of the Spectaculed Salamander, *Salamandrina terdigitata* (Urodela: Salamandridae), Supports the Existance of Two Distinct Species.” *Zootaxa* 995(May):1–19.

McReynolds, Suearl Bullock. 1968. “Effects of Temperature on Secondary Sexual Caharcteristics of the Newts *Notophthalmus viridescens* and *Notophthalmus meridionalis.*” Texas Technological College.

Mecham, John S. 1967a. "*Notophthalmus perstriatus* (Striped Newt)." *Herpetological Review* 26(2):103–4.

Mecham, John S. 1967b. "*Notophthalmus viridescens*." *Herpetological Review* 26(2):104.

Mecham, John S. 1968a. "*Notophthalmus meridionalis*." P. 74.1-74.2 in *Catalogue of American Amphibians and Reptiles*. St. Louis, Missouri: Society for the Maerican Amphibians and Reptiles.

Mecham, John S. 1968b. "On the Relationships between *Notophthalmus meridionalis* and *Notophthalmus kallerti*." *Journal of Herpetology* 2(3):121–27.

Mendoza-Quijano, Fernando, Sol de Mayo Mejenes-Lopez, and Javier Barragan-Torres. 2002. "Notophthalmus Meridionalis (Cope, 1880), Una Adicion a La Anfibiofauna de Hidalgo, Mexico." *Boletin de La Sociedad Herpetologica Mexicana* 10(2):53–54.

Milanovich, Joseph R., William E. Peterman, Nathan P. Nibbelink, and John C. Maerz. 2010. "Projected Loss of a Salamander Diversity Hotspot as a Consequence of Projected Global Climate Change." *PLoS ONE* 5(8).

Milanovich, Joseph R., William E. Peterman, Nathan P. Nibbelink, and John C. Maerz. 2010. "Projected Loss of a Salamander Diversity Hotspot as a Consequence of Projected Global Climate Change." 5(8).

Moritz, Craig, Christopher J. Schneider, and David B. Wake. 1992. "Evolutionary Relationships Within the *Ensatina eschscholtzii* Complex Confirm the Ring Species Interpretation." *Syst. Biol* 41(3):273–91. Retrieved (https://theantlife.com/wp-content/uploads/2014/03/Mortiz-etal-1992_Ring-species-interpretation-confirmed-in-salamanders.pdf).

Moulton, D. W., T. E. Dahl, St Petersburg, and D. M. Dall. 1997. "March , 1997 United States Department of the Interior Fish and Wildlife Service Executive Summary Introduction Survey Methods Results and Discussion Conclusions Literature Cited Appendix A : Habitat Categories Appendix B : Data Tables Acknowledgments."

Mullen, Lindy B., H.Arthur Woods, Michael K. Schwartz, Adam J. Sepulveda, and Winsor H. Lowe. 2010. "Scale-Dependent Genetic Structure of the Idaho Giant Salamander (*Dicamptodon aterrimus*) in Stream Networks." *Molecular Ecology* 19(5):898–909.

Musgrove, MaryLynn et al. 2001. "Geochronology of Late Pleistocene to Holocene Speleothemsfrom Central Texas: Implications for Regional Paleoclimate." *Geological Society of America Bulletin* 113(12):1532–43. Retrieved (http://gsabulletin.gsapubs.org/content/113/12/1532.abstract%5Cnhttp://gsabulletin.gsapubs.org/cgi/doi/10.1130/0016-7606(2001)113%3C1532:GOLPTH%3E2.0.CO;2).

Nathan, Lucas R., Megan D. Simmons, Benjamin Wegleitner, Christopher L. Jerde, and Andrew Mahon. 2014. "Quantifying Environmental DNA Signals for Aquatic Invasive Species across Multiple Detection Platforms Department of Biology , Institute for Great Lakes Research , Central Michigan Environmental Change Initiative , Department of Biological

Sciences , Univ." *Environmental Science & Technology* 48:12800–806.

Noël, Sarah, Martin Ouellet, Patrick Galois, and François Joseph Lapointe. 2007. "Impact of Urban Fragmentation on the Genetic Structure of the Eastern Red-Backed Salamander." *Conservation Genetics* 8(3):599–606.

Oliver, G. V., A. H. Chaney, L. Miller, and S. Parker. 1980. *Vertebrates of (the) Boscaje de La Palma (Area of) Extreme Southern Texas*. Unpublished Report to the Lower Rio Grande Valley Refuge, US Fish & Wildlife Service, Alamo, Texas.

Pan, T. et al. 2014. "Range-Wide Phylogeography and Conservation Genetics of a Narrowly Endemic Stream Salamander, *Pachyhynobius shangchengensis* (Caudata, Hynobiidae): Implications for Conservation." *Genetics and Molecular Research* 13(2):2873–85.

Pearson, Richard G., Christopher J. Raxworthy, Miguel Nakamura, and A. Townsend Peterson. 2007. "Predicting Species Distributions from Small Numbers of Occurrence Records: A Test Case Using Cryptic Geckos in Madagascar." *Journal of Biogeography* 34(1):102–17.

Perry, Jennifer C. and Bernard D. Roitberg. 2006. "Trophic Egg Laying: Hypotheses and Tests." *Oikos* 112(3):706–14.

Petranka, J. W. 1998. *Salamanders of the United States and Canada*. Washington and London: Smithsonian Institution Press.

Petranka, J. W. 2010. *Salamanders of the United States and Canada.* Smithsonian Books.

Phillips, Steven J. and Miroslav Dudík. 2008. "Modeling of Species Distribution with Maxent: New Extensions and a Comprehensive Evalutation." *Ecograpy* 31(December 2007):161–75.

Phillips, Steven J., Miroslav Dudik, and Robert E. Schapire. n.d. "Maxent Software for Modeling Species Niches and Distributions (Version 3.4.1)." Retrieved December 7, 2017 (http://biodiversityinformatics.amnh.org/open_source/maxent/).

Pilliod, David S., Caren S. Goldberg, Robert S. Arkle, Lisette P. Waits, and John Richardson. 2013. "Estimating Occupancy and Abundance of Stream Amphibians Using Environmental DNA from Filtered Water Samples." *Canadian Journal of Fisheries and Aquatic Sciences* 70(8):1123–30. Retrieved (http://www.nrcresearchpress.com/doi/abs/10.1139/cjfas-2013-0047).

Pittman, S. E. and R. D. Semlitsch. 2013. "Habitat Type and Distance to Edge Affect Movement Behavior of Juvenile Pond-Breeding Salamanders." *Journal of Zoology* 291(2):154–62.

van Proosdij, André S. J., Marc S. M. Sosef, Jan J. Wieringa, and Niels Raes. 2016. "Minimum Required Number of Specimen Records to Develop Accurate Species Distribution Models." *Ecography* 39(6):542–52.

Raffel, T. R., J. R. Rohr, J. M. Kiesecker, and P. J. Hudson. 2006. "Negative Effects of Changing Temperature on Amphibian Immunity under Field Conditions." *Functional Ecology*

20(5):819–28.

Ramirez-Bautista, Aurelia et al. 2010. *Lista Anotada de Los Anfibios Y Reptiles Del Estado de Hidalgo , México*. Pachuca, Hidalgo.

Ramirez, P.Jr. 1986. *Water Development Projects in the Rio Grande and Their Relationships to the Santa Ana and Rio Grande Valley National Wildlife Refuges*. Corpus Crisit, TX.

Rappole, John H. and John Klicka. 1991. "Status of the Black-Spotted Newt (Notophthalmus Meridionalis) in Texas and Mexico." Unpublished Report to the Lower Rio Grande Valley Wildlife Refuge, U.S. Fish and Wildlife Service, Alamo, Texas.

Rappole, John H., Charles E. Russell, James R. Norwine, and Timothy E. Fulbright. 1986. "Anthropogenic Pressures and Impacts on Marginal, Neotropical, Aemiarid Ecosystems: The Case of South Texas." *The Science of the Total Environment* 55:91–99.

Reese, Robert W. and I.Lester Firschein. 1950. "Herpetological Results of the University of Illinois Field Expedition , Spring 1949 II. Amphibia." *Transactions of the Kansas Academy of Science* 53(1):44–54.

Reilly, Sean B. and David B. Wake. 2015. "Cryptic Diversity and Biogeographical Patterns within the Black Salamander (*Aneides flavipunctatus*) Complex." *Journal of Biogeography* 42(2):280–91.

Reilly, Stephen M. 1990. "Biochemical Systemics and Evolution of the Eastern North American Newts, Genus Notophthalmus (Caudata: Salamandridae)." *Herpetologica* 46(1):51–59.

Renan, Sharon et al. 2017. "Living Quarters of a Living fossil—Uncovering the Current Distribution Pattern of the Rediscovered Hula Painted Frog (*Latonia nigriventer*) Using Environmental DNA." *Molecular Ecology* 26(24):6801–12.

Rissler, Leslie J., Joseph J. Apodaca, and John Weins. 2007. "Adding More Ecology into Species Delimitation: Ecological Niche Models and Phylogeography Help Define Cryptic Species in the Black Salamander (*Aneides flavipunctatus*)." *Systematic Biology* 56(6):924–42. Retrieved (http://academic.oup.com/sysbio/article/56/6/924/1655548/Adding-More-Ecology-into-Species-Delimitation).

Rius, Marc and John A. Darling. 2014. "How Important Is Intraspecific Genetic Admixture to the Success of Colonising Populations?" *Trends in Ecology and Evolution* 29(4):233–42. Retrieved (http://dx.doi.org/10.1016/j.tree.2014.02.003).

Robinson, D. 1993. "Effects of Reduced Ambient pH on Sodium Balance in the Red-Spotted Newt, *Notophthalmus viridescens*." *Physiological Zoology* 66(4):602–18.

Rodrigues, João Fabrício Mota, Marco Túlio Pacheco Coelho, and José Alexandre Felizola Diniz-Filho. 2016. "Exploring Intraspecific Climatic Niche Conservatism to Better Understand Species Invasion: The Case of *Trachemys dorbigni* (Testudines, Emydidae)." *Hydrobiologia* 779(1):127–34.

Roe, Andrew and Kristine Grayson. 2009. “Repeated Exposure to Fluorescent Powder Does Not Affect Survival or Mass in Eastern Red-Spotted Newts, *Notophthalmus viridescens*.” *Applied Herpetology* 6:295–99.

Roe, Andrew W. and Kristine L. Grayson. 2008. “Terrestrial Movements and Habitat Use of Juvenile and Emigrating Adult Eastern Red-Spotted Newts, *Notophthalmus viridescens*.” *Journal of Herpetology* 42(1):22–30.

Rosmarino, Nicole J. and James J. Tutchton. 2007. *A Petition to List All Critically Imperiled or Imperiled Species in the Southwest United States as Threated or Endangered Under the Endangered Species Act 16 U.S.C. §§ 1531 et Seq.* Santa Fe, New Mexico.

Sawyer, Sarah C., Clinton W. Epps, and Justin S. Brashares. 2011. “Placing Linkages among Fragmented Habitats: Do Least-Cost Models Reflect How Animals Use Landscapes?” *Journal of Applied Ecology* 48(3):668–78.

Schmidt, Karl P. 1953. *A Check List of North American Amphibians and Reptiles*. Sixth. Chicago, IL: The University fo Chicago Press.

Scott, Norman J.Jr. 1996. “Evolution and Management of the North American Grassland Herpetofauna.” Pp. 40–53 in *Ecosystem Disturbance and Wildlife Conservation in Western Grasslands*, edited by D. M. Finch. Albuquerque, NM: USDA Forest Service.

SEMARNAT. 2002. *Norma Oficial Mexicana. Nom-059-Ecol-2001. Protección Ambiental-Especies Nativas de México de Flora Y Fauna Silvestres-Categorias de Riesgo Y Especificaciones Para Su Inclusión, Exclusión O Cambio-Lista de Especies En Riesgo*. Retrieved (http://www.biodiversidad.gob.mx/pdf/NOM-059-ECOL-2001.pdf).

Semlitsch, R. D., K. M. O’Donnell, and F. R. Thompson III. 2014. “Abundance, Biomass Production, Nutrient Content, and the Possible Role of Terrestrial Salamanders in Missouri Ozark Forest Ecosystems.” *Canadian Journal of Zoology* 92(October):997–1004.

Shafer, Aaron B. A., Catherine I. Cullingham, Steeve D. Côté, and David W. Coltman. 2010. “Of Glaciers and Refugia: A Decade of Study Sheds New Light on the Phylogeography of Northwestern North America.” *Molecular Ecology* 19(21):4589–4621.

Shaffer, H.Bradley and Mark L. McKnight. 1996. “The Polytypic Species Revisited: Genetic Differentiation and Molecular Phylogenetics of the Tiger Salamander, *Ambystoma tigrinum* (Amphibia: Caudata), Complex.” *Evolution* 50(1):417–33. Retrieved (http://www.jstor.org/stable/2410811%5Cnhttp://www.jstor.org/stable/pdfplus/2410811.pdf?acceptTC=true).

Smith, Hobart M. and Edward H. Taylor. 1948. “An Annotated Checklist and Key to the Amphibia of Mexico.” *Bulletin of the U. S. National Museum* 194:iv + 118 p.

Smith, Rebecca, Angy Chambers, Joan Berish, Tracey Tuberville, and Kris Herpich. 2005. “Gopher Tortoise Burrow Surveys: External Characteristics, Burrow Cameras, and Truth.” *Applied Herpetology* 2(2):161–70.

De Souza, Lesley S., James C. Godwin, Mark A. Renshaw, and Eric Larson. 2016. "Environmental DNA (eDNA) Detection Probability Is Influenced by Seasonal Activity of Organisms." *PLoS ONE* 11(10):1–15. Retrieved (http://dx.doi.org/10.1371/journal.pone.0165273).

Starkey, David E. et al. 2003. "Molecular Systematics, Phylogeography, and the Effects of Pleistocene Glaciation in the Painted Turtle (*Chrysemys picta*) Complex." *Evolution* 57(1):119–28.

Stebbins, Robert C. 2003. *Western Reptiles and Amphibians*. 3rd ed. New York, New York: Houghton Mifflin Company.

Steele, Craig A. 2006. "Speciation, Phylogeography, and Gene Flow in Giant Salamanders (Dicamptodon)." Washington State University.

Steen, David and James Gibbs. 2005. "Potential Influence of the Terrestrial Landscape on the Distribution of Pond-Breeding Eastern Newts, *Notophthalmus viridescens*." *Applied Herpetology* 2:425–28.

Stein, BA, L. Kutner, and J. Adams, eds. 2000. *Precious Heritage:The Status of Biodiversity in the United States*. Oxford: Oxford University Press.

Stejneger, Leonhard and Thomas Barbour. 1917. *A Check List of North American Amphibians and Reptiles.* Cambridge: Harvard University Press.

Stoeckle, Bernhard C. et al. 2017. "A Systematic Approach to Evaluate the Influence of Environmental Conditions on eDNA Detection Success in Aquatic Ecosystems." *PLoS ONE* 12(12):1–16.

Stohlgren, T. J., C. S. Jarnevich, W. E. Esaias, and J. T. Morisette. 2011. "Bounding Species Distribution Models." *Current Zoology* 57(5):642–47. Retrieved (http://www.scopus.com/inward/record.url?eid=2-s2.0-79961164034&partnerID=40&md5=3b21b87781851afaa308d0b20a83b16b).

Strecker, John K. 1908. "The Reptiles and Batrachians of Victoria and Refugio Counties, Texas." *Proceedings of the Biolological Society of Washington* 21:47–52. Retrieved December 28, 2015 (http://direct.biostor.org/reference/3903).

Strecker, John K. 1922. "An Annotated Catalogue of the Amphibians and Reptiles of Bexar County, Texas." *Science Society of San Antonio* 4:1–31.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL and Waller RW Fischman DL. 2004. "Status and Trends of Amphibian Declines and Extinctions Worldwide." *Science* VOL 306(2002):1783–1786. Retrieved (http://www.natureserve.org/library/gaa_science120304.pdf).

Taggart, T. W. 1997. "Geographic Distribution: *Notophthlamus meridionalis* (Black-Spotted Newt)." *Herpetological Review* 28:47.

Takahara, Teruhiko, Toshifumi Minamoto, Hiroki Yamanaka, Hideyuki Doi, and Zen'ichiro Kawabata. 2012. "Estimation of Fish Biomass Using Environmental DNA." *PLoS ONE* 7(4):3–10.

Taylor, Edward H. 1949. "A Preliminary Account of the Herpetology of the State of San Luis Potosi, Mexico." *University of Kansas Science Bulletin* 33(1):169–216.

Teran-Juarez, Sergio A., Eli Garcia-Padilla, Vicente Mata-Silva, Jerry D. Johnson, and Larry David Wilson. 2016. "The Herpetofauna of Tamaulipas, Mexico: Composition, Distribution, and Conservation Status." *Mesoamerican Herpetology* 3(1):42–113.

Texas Parks and Wildlife. n.d. "Federal and State Listed Amphibians and Reptiles in Texas." Retrieved October 2, 2017 (https://tpwd.texas.gov/huntwild/wild/wildlife_diversity/nongame/listed-species/amphibians-reptiles.phtml).

Thomsen, Philip Francis et al. 2016. "Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes." *PLoS ONE* 11(11):1–22.

Thornton, O. W. 1977. "The Impact of Man upon Herpetological Communities in the Lower Rio Grande Valley, Texas." Texas A&M University , College Station, Texas.

Tiago, Patrícia, Henrique M. Pereira, and César Capinha. 2017. "Using Citizen Science Data to Estimate Climatic Niches and Species Distributions." *Basic and Applied Ecology* 20:75–85. Retrieved (http://dx.doi.org/10.1016/j.baae.2017.04.001).

Tkaczyk, Filip. 2015. *Tracks & Signs of Reptiles and Amphibians: A Guide to North American Species*. First. Mechanicsburg, PA: Stackpole Books.

Turner, Cameron R., Karen L. Uy, and Robert C. Everhart. 2015. "Fish Environmental DNA Is More Concentrated in Aquatic Sediments than Surface Water." *Biological Conservation* 183:93–102. Retrieved (http://dx.doi.org/10.1016/j.biocon.2014.11.017).

U.S. Geological Survey, National Geospatial-Intelligence Agency, and National Aeronautics and Space Administration. 2000. "Shuttle Radar Topography Mission 1 Arc-Second Global, Version 3." Retrieved (https://lta.cr.usgs.gov/SRTM1Arc).

Urban, Mark C., Jonathan L. Richardson, and Nicole A. Freidenfelds. 2014. "Plasticity and Genetic Adaptation Mediate Amphibian and Reptile Responses to Climate Change." *Evolutionary Applications* 7(1):88–103.

US Census Bureau. 2010. "United States Census 2010." *Census.gov*. Retrieved February 20, 2017 (https://www.census.gov/2010census/popmap/index.php).

Valentini, Alice et al. 2016. "Next-Generation Monitoring of Aquatic Biodiversity Using Environmental DNA Metabarcoding." *Molecular Ecology* 25(4):929–42.

Waddle, J. H. 2006. "Use of Amphibians As Ecosystem Indicator Species." University of

Florida.

Wainwright, S. E., M. A. Mora, J. L. Sericano, and P. Thomas. 2001. “Chlorinated Hydrocarbons and Biomarkers of Exposure in Wading Birds and Fish of the Lower Rio Grande Valley, Texas.” *Archives of Environmental Contamination and Toxicology* 40(1):101–11.

Walls, Susan C., Secret S. Belanger, and Andrew R. Blaustein. 1993. “Morphological Variation in a Larval Salamander: Dietary Induction of Plasticity in Head Shape.” *Oecologia* 96(2):162–68.

Walton, B. Michael. 2013. Top-Down Regulation of Litter Invertebrates by a Terrestrial Salamander. *Herpetologica* 69(2):127–46.

Walton, B. Michael, Dimitrios Tsatiris, and Mary Rivera-Sostre. 2006. “Salamanders in Forest-Floor Food Webs: Invertebrate Species Composition Influences Top-down Effects.” *Pedobiologia* 50(4):313–21.

Ward, Darren F. 2007. “Modelling the Potential Geographic Distribution of Invasive Ant Species in New Zealand.” *Biological Invasions* 9(6):723–35.

Weisrock, David W. et al. 2006. “A Molecular Assessment of Phylogenetic Relationships and Lineage Accumulation Rates within the Family Salamandridae (Amphibia, Caudata).” *Molecular Phylogenetics and Evolution* 41(2):368–83.

Weisrock, David W., J. Robert Macey, Ismail H. Ugurtas, Allan Larson, and Theodore J. Papenfuss. 2001. “Molecular Phylogenetics and Historical Biogeography Among Salamandrids of The ‘True’ Salamander Clade: Rapid Branching of Numerous Highly Divergent Lineages in Mertensiella Luschani Associated with the Rise of Anatolia.” *Molecular Phylogenetics and Evolution* 18(3):434–48.

Werner, Earl E. and Mark A. McPeek. 1994. “Direct and Indirect Effects of Predators on Two Anuran Species along an Environmental Gradient.” *Ecology* 75(5):1368–82.

Wheat, Rachel E., Jennifer M. Allen, Sophie D. L. Miller, Christopher C. Wilmers, and Taal Levi. 2016. “Environmental DNA from Residual Saliva for Efficient Noninvasive Genetic Monitoring of Brown Bears (*Ursus arctos*).” *Plos One* 11(11):e0165259. Retrieved (http://dx.plos.org/10.1371/journal.pone.0165259).

Whitmore, S.Scott, Selena Losee, Laurel Meyer, and Theresa A. Spradling. 2013. “Conservation Genetics of the Central Newt (*Notophthalmus viridescens*) in Iowa: The Importance of a Biogeographic Framework.” *Conservation Genetics* 14(4):771–81.

Wilbur, Henry M., Peter J. Morin, and Reid N. Harris. 1983. “Salamander Predation and the Structure of Experimental Communities: Anuran Responses.” *Ecology* 64(6):1423–29.

WildEarth Guardians. 2010. “Fighting for Survival: Black-Spotted Newt.” Retrieved October 1, 2017

(http://www.wildearthguardians.org/site/PageServer?pagename=species_amphibians_black_spotted_newt#.WdqgOGhSy70).

Williams, S.Tyler, Sylvia D. Powell, Thomas A. Gorman, and Carola A. Haas. 2014. "Pioneering the Utility of a Mineral Oil Mixture for Fluorescent Powder Tracking of Salamanders." *Herpetological Review* 45(1):20–22.

Wilson, Larry David and James R. McCranie. 2003. "Herpotofaunal Indicator Species as Measures of Environmental Stability in Honduras." *Caribbean Journal of Science* 39(1):50–67.

Wilting, Andreas et al. 2010. "Modelling the Species Distribution of Flat-Headed Cats (*Prionailurus Planiceps*), an Endangered South-East Asian Small Felid." *PLoS ONE* 5(3).

Wisz, M. S. et al. 2008. "Effects of Sample Size on the Performance of Species Distribution Models." *Diversity and Distributions* 14(5):763–73.

Wolterstorff, W. 1925. "Katalog Der Amphibien-Sammlung Im Museum Fur Natur- Und Heimatkunde Zu Magdeburg." *Abhandlungen Und Berichte Aus Dem Museum Für Natur- Und Heimatkunde Zu Magdeburg* 4:231–310.

Wolterstorff, W. 1930. "Beitrage Zur Herpetologie Mexikos. II. Zur Systemitk Und Biologie Der Urodelen Mexikos." *Abhandlungen Und Berichte Aus Dem Museum Für Natur- Und Heimatkunde Zu Magdeburg* 6(2):129–49.

Wolterstorff, W. 1931. "Uber *Diemictylus kallerti*." *Zoologischer Anzeiger* 94(1/2):13–17.

Wooten, Jessica A., Carlos D. Camp, and Leslie J. Rissler. 2010. "Genetic Diversity in a Narrowly Endemic, Recently Described Dusky Salamander, *Desmognathus folkertsi*, from the Southern Appalachian Mountains." *Conservation Genetics* 11(3):1–20.

Wyman, Richard L. 1988. "Soil Acidity and Moisture and the Distribution of Amphibians in Five Forests of Southcentral New York." *Copeia* 2(2):394–99.

Wyman, Richard L. 1998. "Experimental Assessment of Salamanders as Predators of Detrital Food Webs: Effects on Invertebrates, Decomposition and the Carbon Cycle." *Biodiversity and Conservation* 7(5):641–50.

Wyman, Richard L. and Jeannette Jancola. 1992. "Degree and Scale of Terrestrial Acidification and Amphibian Community Structure." *Journal of Herpetology* 26(4):392–401.

Yap, Tiffany A., Natalie T. Nguyen, Megan Serr, Alexander Shepack, and Vance T. Vredenburg. 2017. "Batrachochytrium Salamandrivorans and the Risk of a Second Amphibian Pandemic." *EcoHealth* 1–14.

York, Patricia et al. 2011. "A Habitat Overlap Analysis Derived from Maxent for Tamarisk and the South-Western Willow Flycatcher." *Frontiers of Earth Science* 5(2):120–29.

Young, Steve and Salvador Sanchez. 2006. *Border 2012: U.S. - Mexico Environmental Program, Indicators Report 2005*.

Yuan, Zhiyong et al. 2018. "Widespread Occurrence of an Emerging Fungal Pathogen in Heavily Traded Chinese Urodelan Species." *Conservation Letters* (December 2017):1–8.

Zhang, Peng, Theodore J. Papenfuss, Marvalee H. Wake, Lianghu Qu, and David B. Wake. 2008. "Phylogeny and Biogeography of the Family Salamandridae (Amphibia: Caudata) Inferred from Complete Mitochondrial Genomes." *Molecular Phylogenetics and Evolution* 49(2):586–97. Retrieved (http://dx.doi.org/10.1016/j.ympev.2008.08.020).

Zhang, Peng and David B. Wake. 2009. "Higher-Level Salamander Relationships and Divergence Dates Inferred from Complete Mitochondrial Genomes." *Molecular Phylogenetics and Evolution* 53(2):492–508.

Zinenko, Oleksandr et al. 2015. "Mitochondrial Phylogeny Shows Multiple Independent Ecological Transitions and Northern Dispersion despite of Pleistocene Glaciations in Meadow and Steppe Vipers (*Vipera ursinii* and *Vipera renardi*)." *Molecular Phylogenetics and Evolution* 84:85–100. Retrieved (http://dx.doi.org/10.1016/j.ympev.2014.12.005).

APPENDIX A

APPENDIX A

SOFTWARE PACKAGES USED AND THEIR SOURCES

Software Title	*Manufacturer's Website*
ArcGIS 10.3	desktop.arcgis.com
GENEIOUS 11.0.1	geneious.com
Mr. Bayes 3.2	mrbayes.sourceforge.net
PartitionFinder 2	robertlanfear.com/partitionfinder
RAxML	github.com/stamatak/standard-RAxML
R 3.3.3	r-project.com

APPENDIX B

APPENDIX B

SURVEY SITES.

Historically active sites marked (^{H}). Individuals found at marked sites (*).

Site	Locations	Start Date	Survey Techniques
*Palo Alto National Battlefield (PANB)**	2	Mar-16	Cover boards (3 arrays of 5 boards, each of size 1.5m x 1.5m x 1cm), debris search, borescope
*Southmost Nature Conservancy (SMNC)^{H}**	2	Sep-15	Cover boards (3 arrays of 5 boards, each of size 0.5m x 0.5m x 1cm), debris search, pitfall trap arrays, artificial burrows (4- 1m in depth and 4- 0.5m in depth)
*Sabal Palm Sanctuary (SPS)^{H}**	4	Sep-15	Cover boards (2 arrays of 5 boards, each of size 0.5m x 0.5m x 1cm), debris search, pitfall trap arrays
*Sal del Rey (SdR)**	2	Dec-16	Debris search
*Laguna Atascosa National Wildlife Refuge (LANWR)^{H}**	12	Mar-16	Cover boards (3 arrays of 3 boards, each of size 1.5m x 1.5m x 1cm), debris search, borescope
Welder Wildlife Refuge ^{H}	6	Apr-16	Debris search, Minnow Traps
Resaca de la Palm State Park (RdlP)	3	Dec-15	Cover boards (2 arrays of 5 boards, each of size 0.5m x 0.5m x 1cm), debris search
Vattmannville^{H}	4	Mar-16	Cover boards (2 arrays of 5 boards, each of size 1.5m x 1.5m x 1cm), debris search
Riviera^{H}	1	Mar-16	Cover boards (1 array of 3 boards, each of size 1.5m x 1.5m x 1cm), debris search
*Willacy**	1	Mar-16	Cover boards (1 array of 5 boards, each of size 0.5m x 0.5m x 1cm), debris search, borescope
La Joya	3	Mar-16	Cover boards (1 array of 10 boards and 2 of 5 boards, each of size 1.5m x 1.5m x 1 cm), debris search

APPENDIX C

APPENDIX C

ILLUMINA SEQUENCE DATA

Newt Sample	*Total Sequences*	*Subset Sequences*	*Max Coverage*	*Min Coverage*	*Average Coverage*	*Haplotype*
Port Isabel 2	4,526,878	607,216	9,089	19	4,140	MER1
Port Isabel 4	5,449,312	598,701	6,234	26	3,233	MER2
Hidalgo 3	10,720,146	1,484,678	20,768	144	10,585	MER3
Hidalgo 6	5,714,886	582,213	6,627	31	2,947	MER3
Laguna Atascosa 1	10,514,832	1,424,431	13,174	22	3,840	MER1
Laguna Atascosa 2	5,589,214	807,362	11,902	61	6,171	MER4
Palo Alto	9,895,124	1,066,946	14,244	31	5,250	MER5
Altamira 3	4,519,964	419,518	5,774	38	2,465	KAL1
Altamira 1	24,552,794	3,120,909	36,199	285	15,165	KAL2
Altamira 4	8,091,612	1,148,059	17,336	104	5,985	KAL1

BIOGRAPHICAL SKETCH

Evan A. Bare graduated from the University of California, Davis in 2015 with a Bachelor of Science degree in Evolution, Ecology, and Biodiversity. During his time at Davis, Evan participated in multiple research projects including studies on the federally endangered California Tiger Salamander (*Ambystoma californiense*) and Cascades frog (*Rana cascadae*). After graduating, Evan continued to pursue research and assisted in Pacific Rattlesnake (*Crotalus oreganus*) research before being accepted to the University of Texas Rio Grande Valley in August 2015 to earn his Master's of Science in Biology. Evan has been accepted to a doctoral program at Trent University under the supervision of Dr. Dennis Murray. Starting in July 2017, Evan will be studying dispersal behaviors and road ecology of endangered tiger salamanders on Pelee Island. Evan can be contacted at evanabare@gmail.com.