Final Report

MONITORING DIVERSITY OF TEXAS TORTOISE POPULATIONS AND DISTRIBUTION OF THEIR RISKS



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(Principal Investigators: Shawn F. McCracken, Candice Y. Lumibao)

by

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS 2

CHAPTER I: BACKGROUND INFORMATION 5

CHAPTER II: COMPARISON OF TEF1 GENE 8

METHODS 8

RESULTS 12

CHAPTER III: MAP OCCURRENCE OF TEXAS TORTOISES THROUGHOUT RANGE AND ASSESS STATUS OF NSD 18

METHODS 18

RESULTS 20

CHAPTER IV: ASSESS IMPACTS OF GENETIC DIVERSITY 26

METHODS 26

RESULTS 27

CHAPTER V: DISCUSSION 30

FUTURE RESEARCH RECOMMENDATIONS 31

LITERATURE CITED 33

EXECUTIVE SUMMARY

The Texas tortoise (*Gopherus berlandieri*) is an understudied species of Greatest Conservation Need, currently receiving no federal protection. However, threats to Texas tortoise populations are present and expanding throughout their range. Increased infrastructure, habitat loss, invasive grasses, and disease all present risks to Texas tortoise populations, fragmenting their populations and compromising their health. To help abate the mortalities development of liquified natural gas facilities and industrial sites may cause, the Federal Energy Regulatory Commission requires new site development to follow Texas Parks and Wildlife Department's Best Management Practices for Species of Greatest Conservation need. For Texas tortoises this includes relocation of individuals away from development. Translocation of tortoises can alter movement patterns and with that, increase the risk of disease communication. With the threat of disease transmission, it is imperative that baseline data on disease presence be collected to facilitate informed relocation of individuals. In this contract, we surveyed Texas tortoise populations throughout their Texas range to monitor the presence of necrotizing scute disease (NSD).

For Task 1 of the contract, our objective was to isolate *Fusarium* spp. from Texas tortoises and sorghum (*Sorghum bicolor*) to compare the similarity of the TEF1 gene and, consequently, whether the same pathogen was causing disease in these hosts. We selected three sites where NSD had been observed on Texas tortoises and one site where it had not been observed. We used active surveys to locate these tortoises (26 total) and swabbed each at least three times. We collected seedheads from fields < 15 km from the tortoise survey sites. We cultured fungal spores from the shell swabs and sorghum seedheads on PDA. We isolated a total of 9 unique *Fusarium* spp. and 1 close relative from three sorghum and seven Texas tortoises. These *Fusarium* spp. isolates spanned four different *Fusarium* species complexes. Based on the phylogenetics, the isolates were no more related to each other based on the host they were isolated from or where they were collected.

For Task 2 of the contract, our objective was to map the presence of Texas tortoises with NSD and sorghum to determine whether proximity to these crops predicted whether Texas tortoises had NSD. We used data collected from the field on Texas tortoises and the community science platform iNaturalist to create a map of where apparent NSD is present throughout the range in Texas. Additionally, the USDA Cropland database was imported into QGIS to analyze the proximity of Texas tortoises to sorghum fields and average annual precipitation received in the area. Based on models, proximity to sorghum fields and average annual precipitation were the best predictors of apparent NSD status, however, it should be noted the R^2 value was low (0.13). Based on the results from Task 1 and 2, it does not seem that *Fusarium* is moving from sorghum to Texas tortoises. Rather, given the breadth of *Fusarium* spp. isolates, it does appear that multiple species may cause this condition and that environmental conditions may play an important role in whether the *Fusarium* can colonize the tortoise's shell.

For Task 3 of the contract, our objective was to sequence Texas tortoises with and without NSD to compare whether individual genetic diversity metrics were indicative of NSD status. We sampled Texas tortoises from across the range in Texas. These samples were sequenced at the Yale Genomics Core lab on the Illumina NovaSeq 6000. We used the aligned and filtered sequences to calculate metrics of individual heterozygosity with vcftools. Based on t-test comparisons, there did not appear to be any difference in heterozygosity based on NSD status.

ACKNOWLEDGEMENTS

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CHAPTER I: BACKGROUND INFORMATION

The threat of fungal diseases to wildlife health is increasingly recognized as they are now attributed to declines in species of bats (Frick et al. 2010; Blehert et al. 2009), frogs (Fisher and Garner 2020; Lips 2016), salamanders (Stegen et al. 2017), and snakes (Lorch et al. 2016). Unlike many bacterial and viral diseases, fungi are often generalists and thus have a broad range of hosts (Olson et al. 2013; Grünwald et al. 2008). Fungi are also incredibly resilient, with spores or saprophyte stages able to survive outside of a host, increasing the risk of transportation into a potentially naïve population (Fisher et al. 2012; Mitchell et al. 2008). These two factors can make fungal pathogens more transmissible and less manageable than other pathogens. Shifts in environmental conditions (Gadre et al. 2022; Seimon et al. 2007), increased globalization (Fisher et al. 2012) and altered population dynamics and distributions of host species often favor increased disease transmission (Plowright 2007). Current evidence suggests that fungal diseases comprise the highest threat of extinction and extirpation to plant and animal hosts (Fischer et al. 2012). Yet, fungal pathogens remain the most neglected pathogen in research and funding (Fischer et al. 2016; Head et al. 2014). This paucity of information on fungal diseases hinders potential management action to assess risk or mitigate future spread.

Necrotizing scute disease (NSD) is a fungal disease that is characterized by fungal mycelia on the shell that typically appears white on a small area but can grow to cover large sections of the shell (Rose et al. 2001; Figure 1). In more advanced cases of NSD, the fungal-caused scute necrosis progresses to the point that the scute appears transparent and may even slough off, leaving the bone exposed (Figure 2). The etiological agent of NSD was identified as *Fusarium semitectum* (synonym = *F. incarnatum*) through isolation of the fungus, culture on a keratin-based agar, inoculating a healthy tortoise with the fungus, and culture from the inoculated tortoise to confirm the pathogen - fulfilling Koch's postulates on disease agent identification (Rose et al. 2001; Koch 1893). Scutes were excised from wild-caught Texas tortoises in South Texas to culture the etiological agent (Rose et al. 2001). Further, this fungus appears to penetrate the scutes of Texas tortoises, as well as human fingernails, but not Box turtles (Smith 2000). During the process of growth on keratin, *F. semitectum* secretes lipases (Smith 2000), which may aid in colonization (Muhsin et al. 1997). However, beyond the initial identification of this fungus and the comparison of growth on different keratin-based structures (Smith 2000), no further research has been conducted in this system.

Despite the lack of research, tortoises with varying degrees of apparent NSD infection continue to appear in the wild. Sightings of NSD are most common (based on iNaturalist observation photos and current survey data) in Cameron, Nueces, Kleberg, Jim Wells, Willacy, San Patricio, and Hidalgo counties. The Lower Rio Grande Valley (Cameron, Willacy, and Hidalgo counties) is predicted to have the most suitable Texas tortoise habitat in the USA (Parandhaman 2015), so the presence of NSD in these localities is cause for concern. When the etiological agent for NSD was described, the authors postulated that cow hooves may be a source of the disease in Texas tortoises (Rose et al. 2001). Although Cattle are raised in the Lower Rio Grande Valley, they are not always near Texas tortoises, and their spread is not wide enough to

explain all instances of apparent NSD encountered in Texas tortoises. However, *F. semitectum* is also known to cause grain mold, root rot, and stalk rot in sorghum (*Sorghum bicolor*), a common cereal crop, and Cotton (*Gossypium* spp.) in south Texas (Prom et al. 2020; Frederiksen and Odvody 2000; Klich 1986) particularly due to the humid conditions that are favorable to fungal growth. Both crops are found in portions of the Texas tortoise range and appear prevalent in areas where NSD is common (USDA Cropland). Furthermore, *F. semitectum* cultured from Texas tortoises successfully grew on Vogel's minimal media, demonstrating a lack of keratin dependence (Smith 2000). Although this disease is thought to be unlikely to cause fatal harm to tortoises (Rose et al. 2001), no long-term studies have been conducted on fatalities or complications related to long-term infection. Further, tortoise shells serve important functions such as predator and pathogen protection (Achrai and Wagner 2017; Nardoni et al. 2012). Degradation of scutes may make tortoises more vulnerable to predation (Achrai and Wagner 2017) and more vulnerable to secondary pathogen invaders (i.e., *Acremonium strictum* seemed to be a secondary invader on *Testudo hermanni* following *F. semitectum* infection, Nardoni et al. 2012).

Shell and fungal diseases in Chelonia are an area of limited research to date, especially in wild animals, despite the risk of mortality due to secondary invaders (Bishop et al. 2010). Pond Turtle Shell Disease is an emerging infectious disease caused by a keratinophilic fungus (*Emydomyces testavorans)* that similarly causes shell lesions (Woodburn et al. 2021). Occasionally, some of these lesions are severe enough to penetrate the coelomic cavity (Woodburn et al. 2021), which raises concerns about how deep NSD may penetrate the shells of Texas tortoises. The first case of *Fusarium* in tortoises was discovered in 1965 in the Radiated tortoise (*Testudo radiata*) (Frank 1965). This *Fusarium*-caused infection resulted in the sudden loss of scutes, but it was only identified using microscopic identification of fruiting bodies to assign to the genus and is not thought to be common (Frank 1965). In other species, *Fusarium* infections can present a larger problem. For example, members of the *Fusarium solani* species complex (FSSC) have been associated with reduced hatchling success in seven globally distributed sea turtles (Sarmiento-Ramírez et al. 2014). In *Caretta caretta*, FSSC has led to mass mortalities of nests in Cape Verde (Sarmiento-Ramírez et al. 2010). Fungi of the FSSC persist in disturbed soil and can colonize eggs laid, likely impeding gas exchange and leading to embryonic stress or death (Sarmiento-Ramírez et al. 2014). This information and research stemming from it have led to improved methods of rearing sea turtle eggs in hatcheries that reduce the risk of disease outbreaks (Hoh et al. 2020). Understanding the sources of *F. semitectum* infecting Texas tortoises is essential to assess the risk NSD poses to this species.

The objectives of this project were to 1) compare TEF1 gene region of *Fusarium semitectum* from Texas tortoise and sorghum hosts to determine if the same species is responsible for disease in taxonomically distinct organisms, 2) map the occurrence of Texas tortoises throughout their range in Texas and assess infection status of NSD with proximity to potential source infection routes from agriculture, and 3) assess whether Texas tortoise population diversity influences disease status.



Figure 1. Texas tortoise with apparent necrotizing scute disease covering portions of the carapace.



Figure 2. Texas tortoise from Oso Bay Wetlands Preserve with NSD missing two marginal scutes, nuchal scute and experiencing abscesses (on left) and bone exposure expanding on caudal scute (on right).

CHAPTER II: COMPARISON OF TEF1 GENE

This chapter addresses Task 1 of the contract. Task 1: Agency will compare genetics of *Fusarium semitectum* from Texas tortoise and sorghum hosts to determine if the same species is responsible for disease in taxonomically distinct organisms.

A. Agency will survey for and capture Texas tortoises throughout their range in Texas.
B. Agency will record biological, health and capture data, and perform fungal swabs for each tortoise captured.
C. Agency will collect fungi from sorghum grown in proximity to Texas tortoise samples.
D. Agency will sequence fungal samples from Texas tortoise and sorghum and compare TEF1 gene regions.

METHODS

Task 1A & B: Tortoise Sample Collection.

We conducted tortoise surveys at three sites where NSD has been observed (Laguna Atascosa National Wildlife Refuge [LANWR], Nueces Delta Preserve [NDP], and Oso Bay Wetlands Preserve [OBWP]) and one site where NSD has never been observed (Chaparral Wildlife Management Area [CWMA]) to serve as the control. The three positive sites were paired with a sorghum field located within 11 km of the survey site (Figure 3). We caught all Texas tortoises by hand and followed protocols to avoid undue stress on individual tortoises and prevent the transfer of pathogens. We collected shell swabs in triplicates from at least five individual tortoises per site. For tortoises with signs of NSD, we focused swabbing on infected portions of the shell (Woodburn 2019). We collected an additional swab per tortoise to test the feasibility of direct DNA extraction from the swab. To remove surface contaminants, we first cleaned the swab sites with 70% ethanol. Then, we used a sterile nylon-tipped swab for fungal isolation. We collected swabs from scutes with signs of NSD for 30 seconds while the swab was rolled back and forth (Woodburn 2019). Additionally, we collected scrapings from infected portions of the shell keratin using a sterile dental curette to ensure the infecting agent was isolated (Lambert et al. 2021). We used a student's T-test to compare the success of *Fusarium* isolation from swabs compared to scute scrapings.

Task 1C: Sorghum Sample Collection.

For comparison between hosts, we collected and isolated fungi from sorghum grown in proximity to collected Texas tortoise samples. At each sorghum field, we clipped four seedheads from the panicum of three different plants that appeared to have a fungal infection. To clip these seedheads, we used sterile scissors and flame-sterilized them between each plant. We also changed gloves between each plant to prevent cross-contamination. We then stored these seedheads in a sterile Falcon tube at 4 °C until the samples were plated.

Task 1D: Fungal Cultures.

All lab protocols were approved under IBC Protocol TAMU-CC-IBC-2023-0032-INI-1.0. To make plates for cultures, we followed the protocol for DiFico Potato Dextrose Agar (PDA). We allowed the PDA to solidify under a UV light in a BioSafety Cabinet to prevent contamination before storing them sealed and wrapped in aluminum foil at 4 °C until use. Within 48 hours of sample collection, we plated the biological samples on PDA under a Biosafety Cabinet. To plate the swabs, we used sterile scissors to cut the tip of the swab and gently press it onto the agar on the center of the plate. For the shell scrapings, we used sterile forceps to collect the scraping and place it in the center of the plate. For the sorghum seedheads, we collected only one seed from each seedhead with sterile forceps for culture. We then labeled the plate with the sample code and date and sealed the plate with ParaFilm. Once the plates were sealed, we transferred them to the VWR Incubating Orbital Shaker, which was covered with aluminum foil to prevent light from entering the incubator, and set it to 25 °C. We allowed ~5 days for the fungus to grow. After this point, we checked the plates under a SeBa digital microscope for the presence of multiple fungal morphologies. If more than one fungal morphology was present, we subcultured each morphology onto a new PDA plate. When only one fungal morphology was present, we proceeded to DNA extraction.

Task 1D: DNA Extraction and Quantification.

For each subculture, we used a sterile inoculating loop to collect a small sample of the fungal mycelia and transferred it to a labeled tube while in the BioSafety Cabinet. We then used the Extract-n-Amp Plant kit (Sigma-Aldrich) following a protocol for fungal mycelium DNA extraction (U'Ren et al. 2016). We made the minor modification of inverting the sample tubes every 2 minutes while on the heat block to assist in breaking up the cell walls. We quantified the amount of DNA from each sample using QuBit dsDNA High Sensitivity Assay Kit (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. For samples that had a DNA concentration of < 4 ng/ml, we reextracted the DNA to improve the sample's DNA concentration.

For the swabs left to test direct sequencing methods, we attempted to extract DNA with the Extract-n-Amp Plant kit and the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's protocol. We also attempted to directly extract DNA from these swabs using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following a protocol for sequencing fungi from snake scale swabs (Harding et al. 2022). We used repeat swabs collected from the same two Texas tortoises at Oso Bay Wetlands Preserve to determine which method was the most successful. The success of extraction was based on the DNA concentration from the QuBit dsDNA High Sensitivity Assay Kit. We then applied the top performing method to the remainder of these swabs.

Task 1D: Polymerase Chain Reaction.

To identify the *Fusarium* species affecting the tortoises and sorghum, we targeted the TEF1 gene and internal transcribed spacer (ITS) region for PCR amplification and sequencing. The TEF1 gene is a useful phylogenetic marker with *Fusarium*-specific primers (Karlsson et al. 2016) that frequently provides better *Fusarium* species-level discrimination than the traditional

ITS region (Karlsson et al. 2016; Geiser et al. 2004). However, we amplified both the ITS and TEF1 to allow for multi-locus analyses.

To amplify the ITS region, we used the ITS1-F/LR3 primer set (Bergmann et al. 2022; Gardes and Bruns 1993; Vilgalys and Hester 1990). The master max for the ITS region per sample included 0.3 ml of each primer at 10 mM, 7 ml ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich), and 6.4 ml of PCR water. We mixed the master mix with 1 ml of the extracted DNA. We ran the samples in a Flexlid Mastercycler Nexus Thermal Cycler (Eppendorf, SE, Germany) under the following conditions: 94 °C for 3 mins; 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec, and 72 °C for 1 min; and 10 mins 72 °C for 10 mins. After PCR, we ran the samples on a 1.5% agarose gel to ensure the sample had amplified correctly. If the sample had a band, we proceeded to run a new PCR on the sample to target the TEF1 gene.

We amplified the TEF1 gene using the *Fusarium*-specific primers Fa+7/Ra+6 (Karlsson et al. 2016). The master mix for the TEF1 gene per sample included 0.375 ml of each primer at 10 mM, 7 ml ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich), and 6.25 ml PCR water. We mixed the master mix with 1 ml of the extracted DNA. We ran the samples in the Mastercycler with the conditions of 94 °C for 3 mins; 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min; and 72 °C for 10 mins. Following, we ran the samples on a 1.5% agarose gel to check for the presence of bands at the 500-600 bp mark.

Task 1D: Sequence Analysis.

After PCR, we had the samples Sanger sequenced at the TAMU-CC Genomics Core lab. Prior to sequencing, they purified the samples using EXOSAP-IT. We proof-read and analyzed sequences in Geneious Prime (Dotmatics Version 2025.1.1), followed by BLAST+ analysis against the UNITE database (Abarenkov et al. 2024) for the ITS region and FUSARIOID-ID database for *Fusarium* species identification (Geiser et al. 2004) in Geneious Prime (Dotmatics Version 2025.1.1). We then noted which species complex each sequence fell within and whether this matched with the identification for both barcoding regions.

Task 1D: Phylogenetic Analysis

One of the isolates was closely matched with the genus *Fusicolla*, so we used it as the outgroup, given its distance from the other species complexes represented (Li et al. 2023). With multiple *Fusarium* species complexes represented and a small sample size, we used representative strains from these complexes and other related species complexes gathered from GenBank to build the phylogenetic trees. Many *Fusarium* spp. strains on GenBank were limited in only having the TEF1 gene, so we made a phylogenetic tree using only the TEF1 gene of the isolates from this study, a tree with the ITS region and TEF1 gene with a few representatives from each species complex represented, and a tree with several isolates focused solely on the TEF1 gene. We used MAFFT (version 7.490; Katoh and Standley 2013; Katoh et al. 2002) to align the TEF1 gene and ITS region separately. We checked alignments manually and corrected them as needed. We then exported these alignments as a FASTA file and imported them into ramxl GUI (version 2.0.14; Elder et al. 2021; Kozlov et al. 2019; Stamatakis 2014). We used the

model test feature (Darriba et al. 2020) for each barcode region to determine the substitution model, rate heterogeneity, and stationary frequencies. Following, we concatenated the sequences and ran a maximum likelihood analysis with transfer bootstrap expectation with 1000 repetitions. To create the tree figures, we used the Interactive Tree of Life online interface (Letunic and Bork 2024).

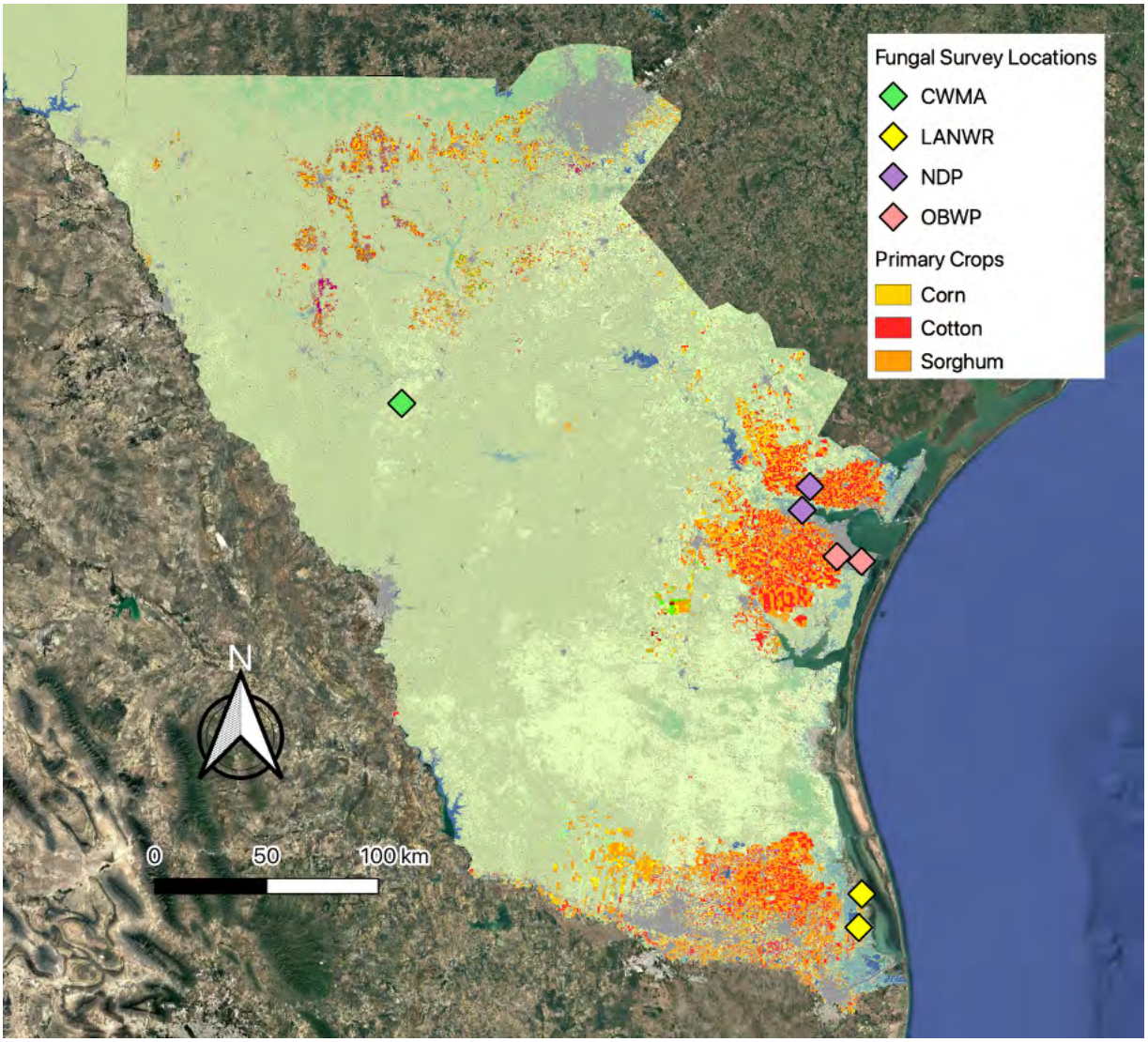


Figure 3. Map of sampling locations displaying land-use type based on the USDA Cropland Database. Orange represents sorghum, yellow represents corn, and red represents cotton. The diamonds represent each of the sampling locations and the corresponding sorghum field sampled.

RESULTS

Task 1A-C: Sample Collection.

A total of 26 tortoises were swabbed to test for the presence of *Fusarium* spp. None of the tortoises at CWMA, the control site, had apparent signs of NSD. No *Fusarium* spp. were isolated from CWMA tortoise shells. At the remaining three sites, 89% of tortoises swabbed (n = 18) had signs of NSD. Of those with signs of NSD, *Fusarium* spp. was successfully isolated from 6 (38% of those with signs) and one from a close *Fusarium* relative (put. *Fusicolla* spp.) (~1% of those with signs). No *Fusarium* spp. were isolated from tortoises without signs of NSD. By site, the total number of isolates are as follows; 4 at OBWP, 2 at NDP, and 1 at LANWR (Table 1).

Samples were collected from a total of 12 sorghum seedheads. Of those tested, *Fusarium* spp. were isolated from 3/12 sorghum seedheads. Two of these seedheads were from NDP and 1 was from OBWP. None of the sorghum seedheads tested at the agricultural field closest to LANWR tested positive for *Fusarium* spp. The ITS sequence data has been submitted to GenBank (submission ID SUB15689064) and the TEF1 sequences data has been submitted to BankIt (submission ID 3012431).

Task 1D: Phylogenetic Analysis

For the phylogenetic tree built with the TEF1 gene with isolates from this study and reference sequences, we used a TIM2 substitution model with a gamma rate heterogeneity and proportion of invariant site because it was the top performing in BIC (score = 6646.36, weight = 0.49), and was competing for top model with AIC (score = 6396.56, weight = 0.11) and AICc (score = 6413.22, weight = 0.29). For the phylogenetic tree built with the TEF1 gene with isolates only from this study, we used a GTR substitution model with a gamma rate heterogeneity because it was the top performing in AIC (score = 3975.66, weight = 0.34) and AICc (score = 3978.67, weight = 0.28). For the phylogenetic tree with the ITS region and TEF1 gene, model testing indicated that the best parameters for the ITS region was TrNef substitution model with a gamma rate heterogeneity (BIC score = 5398.31, BIC weight = 0.77, AIC score = 5253.78, AIC weight = 0.18, AICc score = 5255.78, AICc weight = 0.18) and for the TEF1 gene the best parameters were TIM2 substitution model with a gamma rate heterogeneity and proportion of invariant site (AIC score = 4474.89, AIC weight = 0.33, AICc score = 4479.89, AICc weight = 0.37). There was no indication that fungi were more related based on host or site (Figures 4-6).

A total of 9 isolates were *Fusarium* (or a close relative) across four species complexes, and one from a closely related genus, *Fusicolla* spp. Most commonly (50%), species were isolated from the *Fusarium incarnatum-equiseti* species complex (FIESC). Next, 20% were within the *Fusarium oxysporum* species complex (FOSC). The remaining 30% were divided equally within the *Fusarium fujikuroi* species complex (FFSC), *Fusarium* (=Neocosmospora) *solani* species complex (FSSC), and the genera *Fusicolla* (Figures 5 & 6).

Methodology Comparison.

Between all tortoise shell samples where signs of NSD were present, there was no difference between swabs and shell scrapings and the successful isolation of *Fusarium* spp. (t = -0, p-value = 1). Further, direct sequencing of swabs was not successful. The largest DNA recovery from a directly sequenced swab was 0.5 ng/ul which was insufficient for producing a

band with the ITS1F/LR3 primer set. We did not test direct sequencing degraded keratin scraped from tortoise shells.

ID	Age	Sex	Site	MCL (mm)	Width (mm)	Height (mm)	Weight (g)	Signs of NSD?	Fusarium isolated?	Other health notes?
CWMA01	Adult	Male	CWMA	168	139	84	1105	N	N	NA
CWMA02	Adult	Male	CWMA	191	150	101	1650	N	N	NA
CWMA03	Adult	Female	CWMA	159	139	80	1000	N	N	NA
CWMA04	Adult	Male	CWMA	170	133	85	1075	N	N	Swollen eyelids
CWMA05	Juvenile	Unknown	CWMA	126	105	63	520	N	N	NA
CWMA06	Adult	Female	CWMA	159	134	82	985	N	N	NA
CWMA07	Adult	Female	CWMA	147	128	88	860	N	N	NA
CWMA08	Adult	Female	CWMA	140	130	76	850	N	N	NA
LANWR01	Adult	Male	LANWR	201	166	93	1950	Y	N	
LANWR02	Adult	Male	LANWR	191	155	91	1410	Y	Y	degraded nares, nasal discharge
LANWR03	Subadult	Female	LANWR	145	129	72	710	N	N	degraded nares
LANWR04	Adult	Female	LANWR	162	144	86	1190	Y	N	
LANWR05	Adult	Female	LANWR	162	141	91	930	Y	N	
LANWR06	Adult	Male	LANWR	157	146	90	960	Y	N	
LANWR07	Adult	Male	LANWR	199	168	102	1790	Y	N	
LANWR08	Adult	Male	LANWR	191	152	99	1530	Y	N	
NDP01	Subadult	Female	NDP	143	115	62	530	N	N	Eyes sunken
NDP02	Adult	Female	NDP	164	150	80	1260	Y	N	
NDP03	Adult	Female	NDP	170	144	82	1070	Y	Y	
NDP04	Adult	Female	NDP	182	151	80	1310	Y	Y	Ocular discharge, degraded nares
NDP05	Adult	Male	NDP	180	142	83	1270	Y	N	
OBWP01	Adult	Male	OBWP	218	167	112	1990	Y	Y	
OBWP04	Adult	Female	OBWP	171	147	85	1210	Y	Y	
OBWP07	Adult	Male	OBWP	175	165	90	1130	Y	Y	
OBWP08	Adult	Female	OBWP	151	126	79	990	Y	N	
OBWP09	Adult	Male	OBWP	172	144	86	1090	Y	N	degraded nares

Table 1. Data collected on all the tested Texas tortoises. Measurements in mm refer to the shell, MCL for midline carapace length.

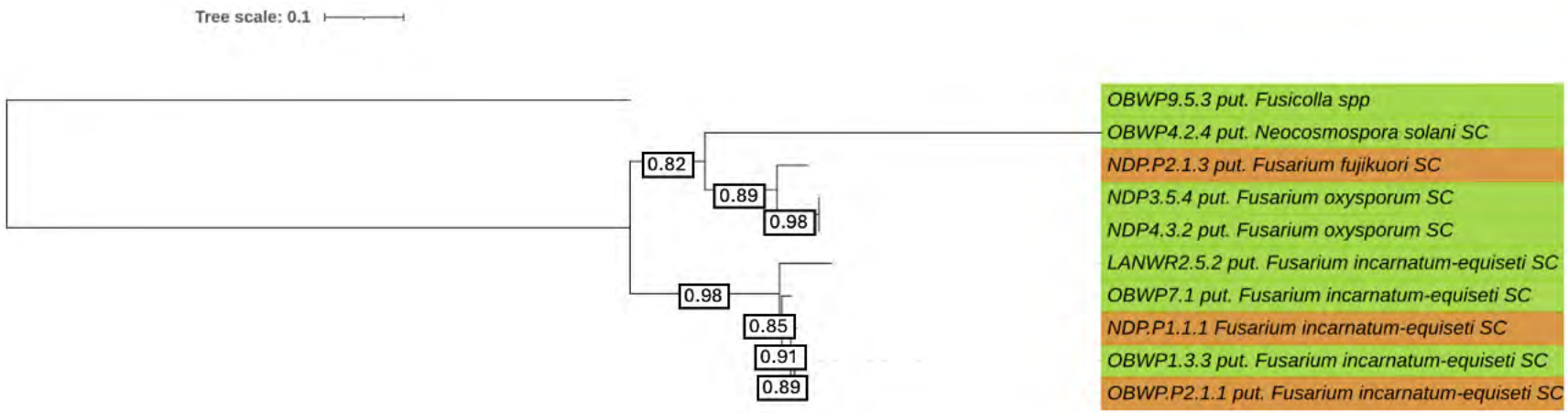


Figure 4. Phylogenetic tree using raxml of the TEF1 gene of the isolates from this study. The colored leaves represent isolates from this study. The orange leaves represent isolates from sorghum (*Sorghum bicolor*) and the leaves in green represent isolates from Texas tortoises (*Gopherus berlandieri).*

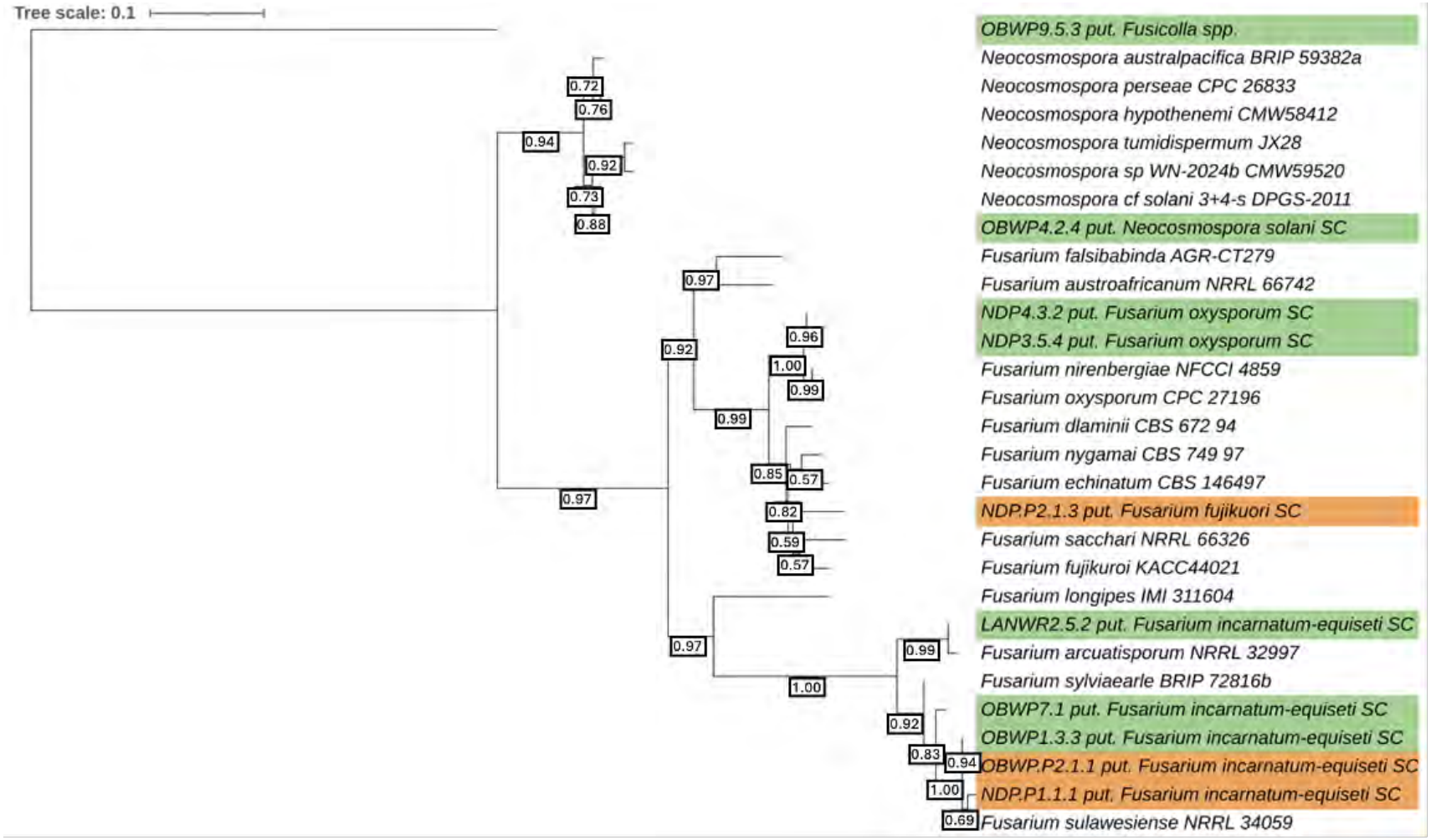


Figure 5. Phylogenetic tree created using raxml of the TEF1 gene of the isolates from this study and representatives from the species complexes represented. The colored leaves represent isolates from this study. The orange leaves represent isolates from sorghum (*Sorghum bicolor*) and the leaves in green represent isolates from Texas tortoises (*Gopherus berlandieri).*

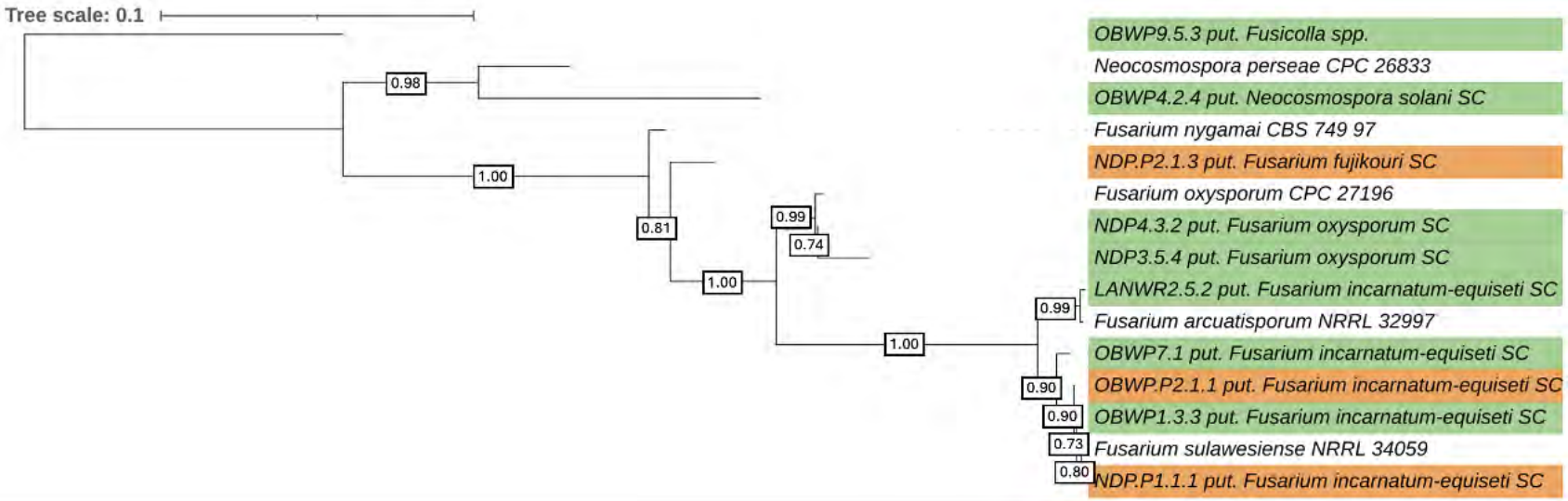


Figure 6. Phylogenetic tree created using raxml of the ITS region and TEF1 gene of the isolates from this study and representatives from the species complexes represented. The colored leaves represent isolates from this study. The orange leaves represent isolates from sorghum (*Sorghum bicolor*) and the leaves in green represent isolates from Texas tortoises (*Gopherus berlandieri)*.

CHAPTER III: MAP OCCURRENCE OF TEXAS TORTOISES THROUGHOUT RANGE AND ASSESS STATUS OF NSD

This chapter addresses Task 2 of the contract. Task 2: Agency will map Texas tortoise occurrence throughout their range in Texas and assess infection status of necrotizing scute disease (NSD) with proximity to potential source infection routes from agriculture.

A. Agency will use a combination of field data from Task 1 and citizen science to acquire location and NSD infection data for Texas tortoises.
B. Agency will map Texas tortoise locations and disease status.
C. Agency will overlay USDA Cropland Data Layer with Texas tortoise locations to compare disease status with proximity to cropland, with emphasis on sorghum, cotton, and corn crops.
D. Agency will assess whether agriculture features predict NSD status.

METHODS

Task 2A: Data Collection.

To map the presence of apparent NSD, we used a combination of field data and community science. To increase the availability of the community sourced observations, we advertised to relevant social media groups (Texas Field Herpers, Texas Master Naturalists, and Texas Turtles; Figure 7) and through public presentations to request that community scientists and biologists upload observations of Texas tortoises to the iNaturalist group (Texas Tortoise Task Force). Participation in this group allowed us to download unobscured coordinates, and given the conspicuous nature of NSD, these observations can be marked for inclusion in the analysis. Where high resolution (< 250 m accuracy) GPS data was available, we included these observations in the analysis. Additionally, some coordinates were included from the Herps of Texas iNaturalist group provided by TPWD. We only used data from tortoises in the USA due to the limitation of high-resolution crop data being unavailable in Mexico.

Task 2A-C: Data Pre-Processing.

Prior to the inclusion of iNaturalist data, we cleaned observations at hotspot points (areas with >40 observations). We compared tortoise observations within a hotspot to ensure multiple observations of the same tortoise were omitted. This omission was done by noting obvious shell markings on all tortoises (blemishes on certain scutes, NSD damage, degree of shell wear) at the site and comparing the images side by side. When the same tortoises were observed, we kept the observation with the highest GPS resolution and removed the rest. We conducted all spatial analyses in QGIS (version 3.26.3).

We used the 2023 Cropland dataset from USDA, which has a resolution of 56 m, to explore the effects of land-use type on the presence of NSD in tortoises. For each county, we converted the Cropland raster into a polygon then extracted pixels corresponding to sorghum,

cotton, corn, and pasture and converted them into new layers. Given that sorghum has the lowest reported accuracy of the agriculture layers (65%; 2023 Texas Cropland Metadata), we manually checked the reported sorghum pixels by cross referencing with Google Earth satellite imagery. Sorghum pixels that were not on an apparent agricultural field (e.g., sorghum pixels overtop of houses) were removed from the layer. For cotton, corn, and pasture/grassland (which had a reported accuracy of 85.3%, 88.6%, and 79.6%, respectively), we used the dissolve feature so all pixels close to each other would become a new polygon. We then calculated the area for each resulting polygon; those polygons smaller than 36 x 36 pixels (or 4 pixels touching), were removed from the layer. This process allowed for erroneous pixels to be removed in an efficient manner.



Figure 7. Advertisement used to encourage people to document Texas tortoises encountered.

Task 2D: Spatial Analysis.

We overlayed survey tortoise data identified by disease status on the Croplands polygon layer. For each Cropland predictor variable, we used the 'shortest distance between feature' tool in QGIS to calculate the distance to each layer. Further, we included average annual precipitation data from 1981 – 2010 (Esri 2019). We conducted the statistical analysis in RStudio (version 4.3.3, R Core Team 2023). Prior to modeling, we created a correlation matrix of the predictor variables and checked the variation inflation factor using the 'usdm' package (Naimi et al. 2014) to ensure there is no collinearity (>0.7) or multicollinearity (VIF > 3) (Zuur et al. 2010). Distance to corn was eliminated as a covariate given the high VIF value (~5) and the primary *Fusarium* spp. causing disease within corn is in a different species complex (Ortiz et al. 2015). All quantitative variables were scaled prior to inclusion in a model. A GLM with a binomial distribution was used to assess whether agriculture features predict NSD status with disease status (NSD present or absent) as the response variable, nearest distances to agriculture types, annual average precipitation, and nearest distance to another Texas tortoise as fixed effects. We used backward stepwise regression and AIC to select the best fit model. We checked the best fit models to ensure there was no overdispersion. We used the 'DHARMa' package to check the residuals of the model (Hartig 2016) and the 'performance' package to check the R^2 values (Lüdecke et al. 2021). Finally, we used the Moran I statistic to test for spatial patterns among the individuals showing signs of NSD using the 'spdep' package (Bivand 2022). The Moran I statistic was weighted by apparent NSD status. The Moran output ranges from -1 to 1, where -1 indicates an even spread across the landscape, 0 indicates random spread, and 1 indicates complete clustering (Cliff and Ord 1969).

RESULTS

Task 2A&B: Mapping Texas tortoises with apparent NSD

After cleaning the iNaturalist data (with data from Task 1 included), a total of 1121 observations of Texas tortoises were included. Of those tortoises, a total of 272 (24.3%) appeared to have NSD. The Moran I statistic for spatial clustering of apparent NSD indicates there is clustering of these tortoises on the landscape (Moran: 0.37, deviance: 8.91, p-value < 0.01, Figure 8). Areas of higher prevalence of apparent NSD include Cameron, Hidalgo, Willacy, Nueces, San Patricio, and Live Oak counties (Figure 8). No apparent NSD is observed in Val Verde, Uvalde, Kinney, Maverick, Bexar, Bee, Zavala, Dimmit, La Salle, Webb, and Zapata counties (Figure 8).

Task 2C&D: Determine whether proximity to agriculture predicts NSD status

The best model for predicting NSD included annual precipitation ($\boldsymbol{\beta}$precip = 0.53 $\pm$ 0.09 SE, z = 6.12, p-value < 0.01), and the distance to sorghum fields ($\boldsymbol{\beta}$sorghum = -0.41 $\pm$ 0.12 SE, z = -3.39, p-value < 0.01) as the predictors. Tortoises were more likely to have NSD as the annual precipitation increased (95% CI: 0.36, 0.70; Figures 9 & 10). As the distance between a tortoise and sorghum field decreased, there was a greater chance the tortoise had NSD (95% CI: -0.64, -0.17; Figures 11 & 12). The Nagelkerke's R^2 value was 0.13.

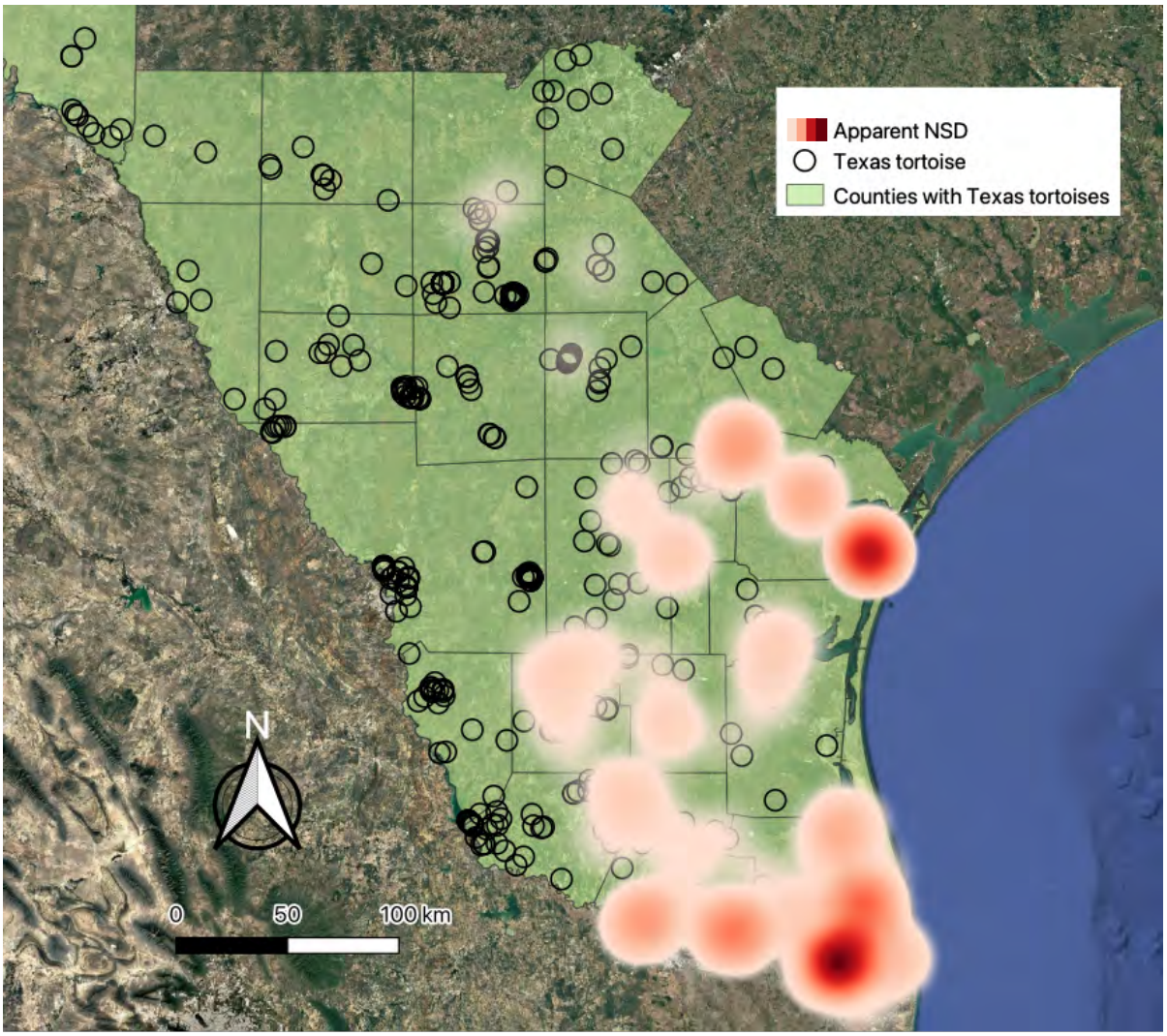


Figure 8. Map of the Texas range (in green) of *Gopherus berlandieri* displaying Texas tortoise individuals with apparent necrotizing scute disease (NSD) in a gradient of light red (one individual) to dark red (several exposed individuals in an area). The black circles indicate individuals that did not show signs of NSD.

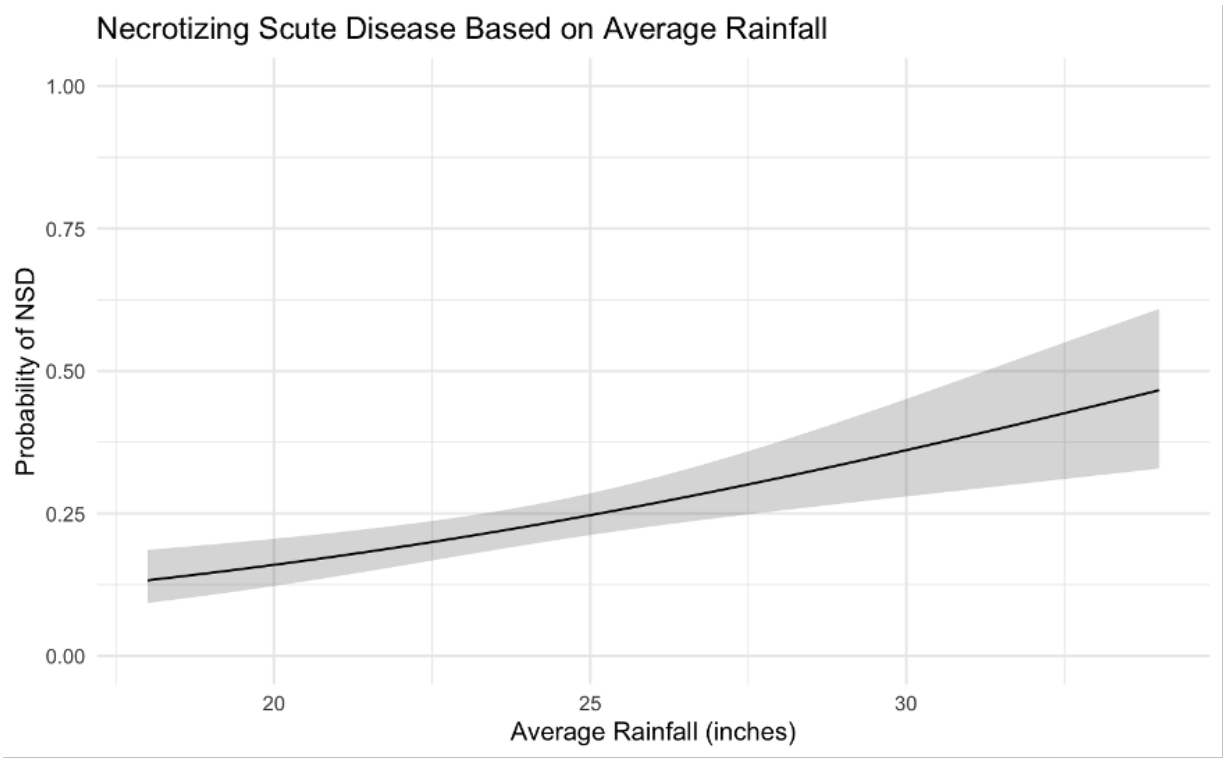


Figure 9. Probability of a Texas tortoise having apparent necrotizing scute disease (NSD) based on average annual precipitation (recorded in inches). The probability of apparent NSD increases as the average precipitation increases. The grey shading represents the 95% confidence intervals. The model was supported with a Nagelkerke's R^2 value of 0.13 and p-value of < 0.01 for the average precipitation variable.

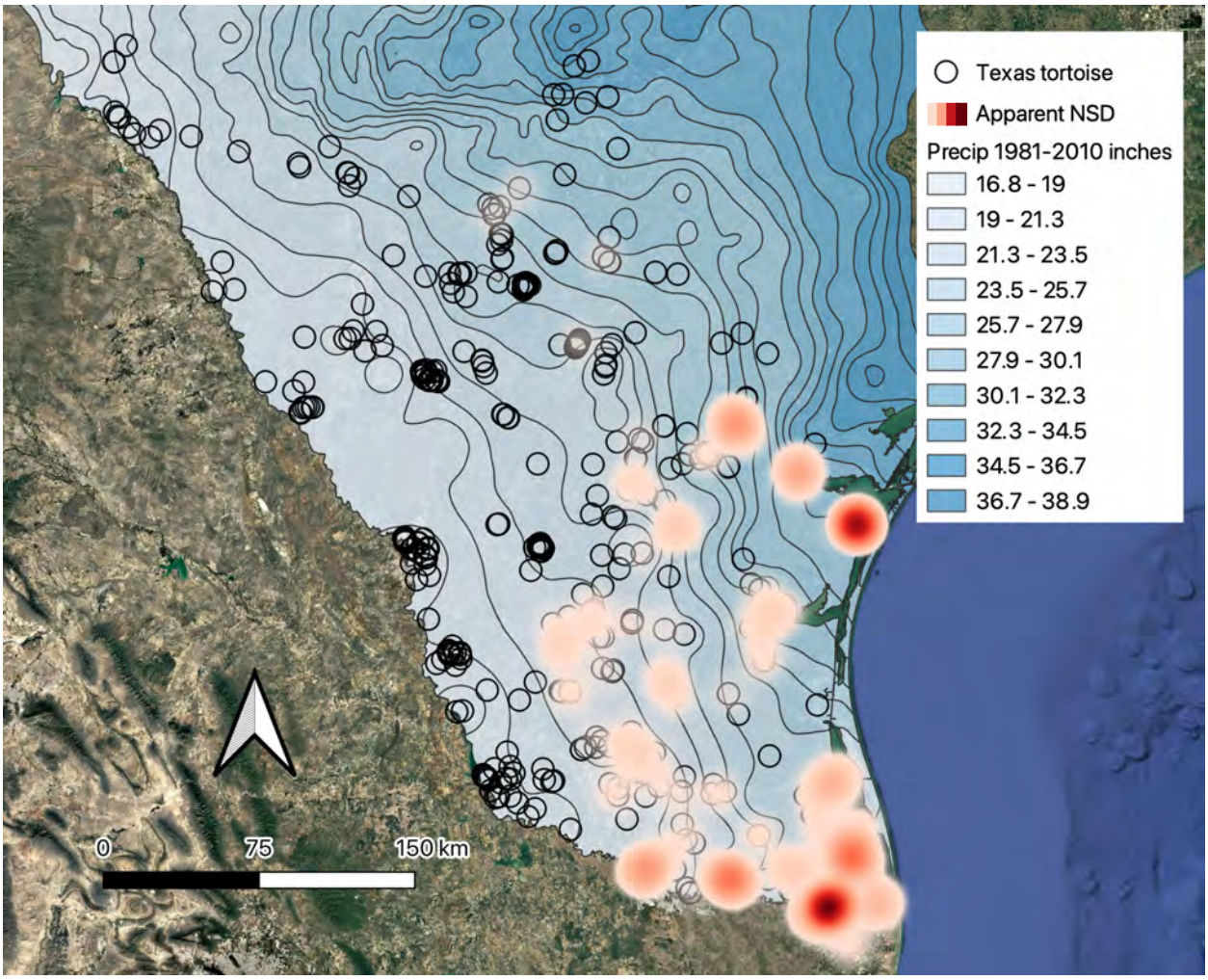


Figure 10. Map of south Texas displaying the precipitation gradient (darker blue indicates more average annual precipitation) in the region with the heatmap of where NSD is apparent vs where tortoises without apparent NSD were detected. The size of the detections for NSD apparent tortoises is reduced to display map features.

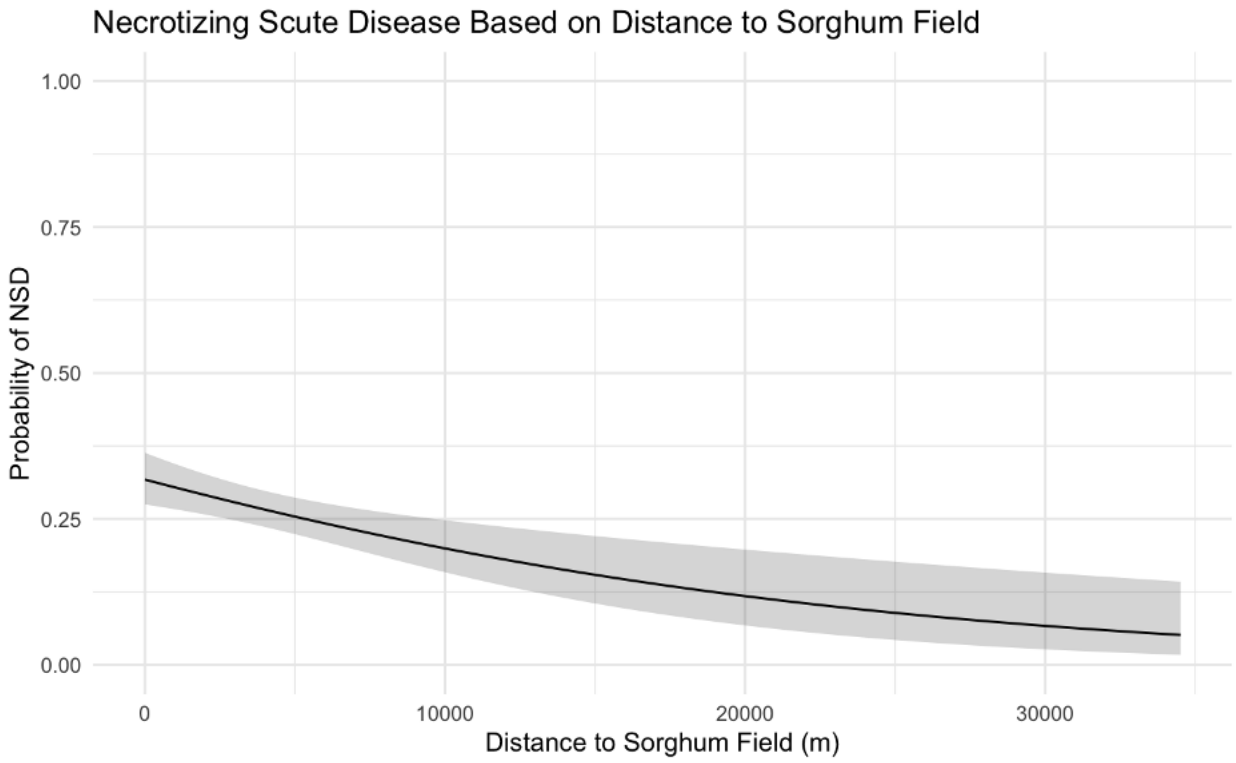


Figure 11. Probability of a Texas tortoise having apparent necrotizing scute disease (NSD) based on the distance to a sorghum field. The probability of apparent NSD decreases as the distance to the nearest sorghum field increases. The grey shading represents the 95% confidence intervals. The model was supported with a Nagelkerke's R^2 value of 0.13 and p-value of < 0.01 for the distance to sorghum field variable.

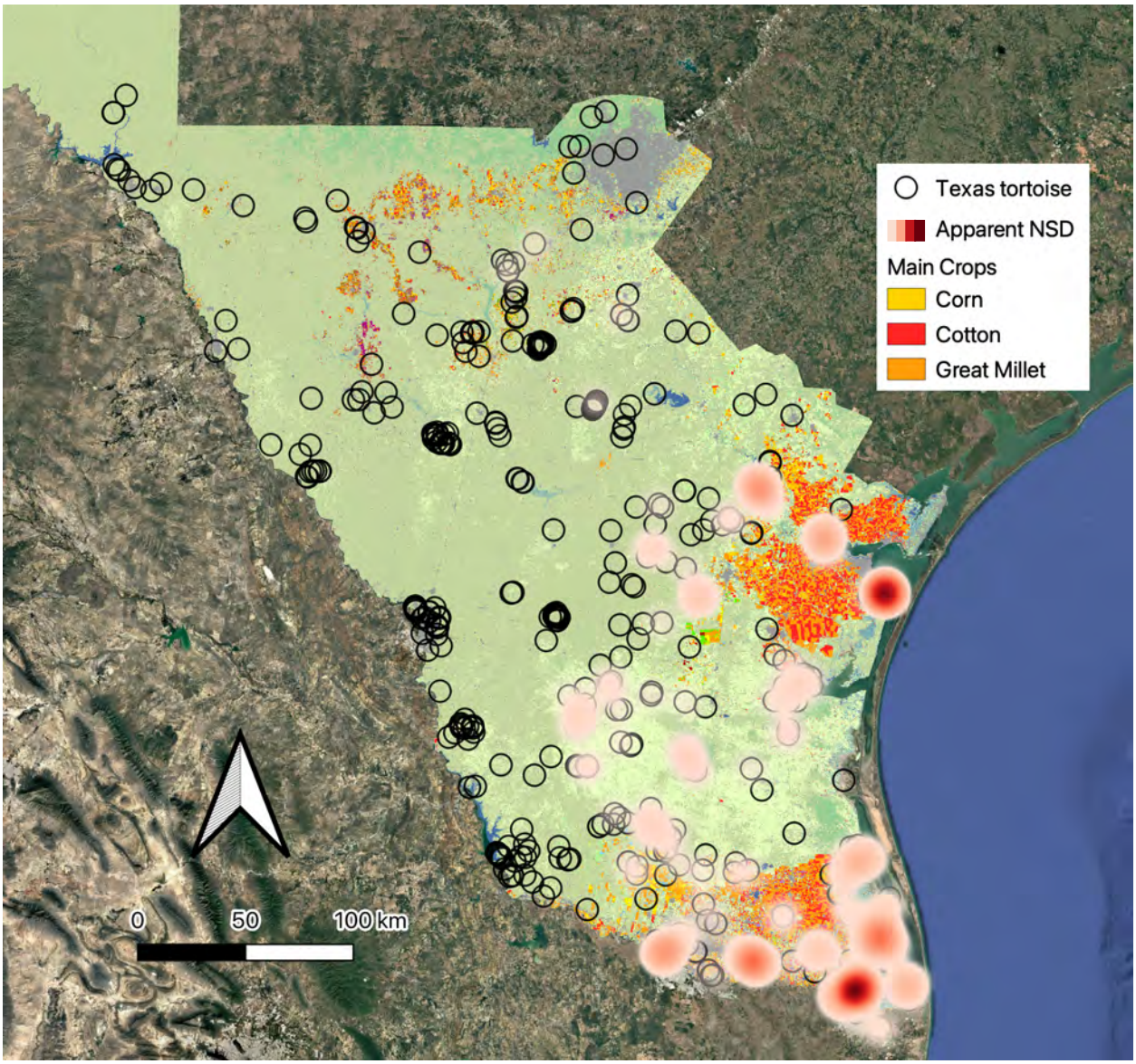


Figure 12. Map of south Texas displaying the USDA Cropland data from the region with the heatmap of where NSD is apparent vs where tortoises without apparent NSD were detected. The size of the detections for NSD apparent tortoises is reduced to display map features.

CHAPTER IV: ASSESS IMPACTS OF GENETIC DIVERSITY

This chapter addresses Task 3 of the contract. Task 3: Agency will assess whether Texas Tortoise population diversity influences disease status.

A. Agency will collect blood samples from up to 15 Texas Tortoises with visually apparent NSD captured in Task 1.
B. Agency will sequence genomes of the Texas Tortoises sampled above.
C. Agency will compare genomes of NSD-infected Texas Tortoises to genomes of uninfected Texas Tortoises.

METHODS

Task 3A: Tortoise Sample Collection

We followed the handling practices outlined in Task 1A. We collected blood samples (<1% body weight) through the subcarapacial or brachial vein with a syringe and needle that had been flushed with EDTA (Invitrogen, AM9261) to prevent coagulation and centrifuged samples on-site to separate plasma/serum from the red blood cells. With the remaining red blood cells, we pipetted in 2 – 5 x volume of RNAlater (Invitrogen, AM7021) to stabilize and protect RNA for later processing. We then placed the samples in a cooler until they could be moved to the -80 C freezer (no more than 48 hours).

Task 3B: Genome Sequencing

The following methods were conducted following the same pipeline from TPWD report prepared by McCracken et al. 2025: We submitted the samples from Task 3A to the Yale Center for Genomic Analysis for processing and sequencing with Illumina Novaseq 6000. The resulting 150 bp paired-end reads were trimmed for adapters using bbduk (bbmap v39.10; Bushnell, 2014) with kmer length of 23 (minimum k length of 11) and allowing 1 mismatch. Then reads were aligned to a reference *Gopherus berlandieri* genome (Baty et al., in prep) with bwa mem v0.7.17 (Li and Durbin, 2009). To guide downstream filtering, we calculated genome-wide sequencing coverage with mosdepth v0.3.8 (Pedersen and Quinlan, 2018). Variants were called using gatk v4.6.1.0 (McKenna et al., 2010) and filtered with vcftools v0.1.16 (Danecek et al., 2011) for a minimum per-sample depth of and mean depth of 5x, maximum per-sample depth and mean depth of 30x, maximum missing data of 10%, minimum quality score of 30 per-site, and minor allele frequency of 0.1. Following the gatk best practices pipeline, we used the high-confidence uncalibrated variants from the first round of variant calling to guide base recalibration in a second round (Van Der Auwera et al., 2013). The resulting gVCF files were merged using GenomicsDBImport and jointly genotyped with GenotypeGVCFs. These variants were again filtered with bcftools v1.21 for minimum quality by depth of 2, minimum mean depth of 4, maximum mean depth of 20, minor allele frequency of 0.05, and maximum missing data of 10% (Danecek et al., 2021). Lastly, we pruned reads for linkage disequilibrium with plink v1.90b6.21 with –indep-pairwise using a window size of 50 kb, a 5 kb step size, and a r2 threshold of 0.2 (Purcell et al., 2007).

Task 3C: Compare genomes of tortoises with and without NSD

Given there is no clear population structure of Texas tortoises in the state (McCracken et al. 2025), we compared individual Texas tortoises for this task. We used observed heterozygosity and Wright's F statistic. We used the 'vcftools –het' function. Observed heterozygosity was calculated as number of heterozygous sites by total number of sites examined. F was calculated as: (expected homozygous sites – observed homozygous sites) / (number of total sites examined – expected homozygous sites). We then ran a Welch's t-test to determine whether these metrics differed based on NSD status and included them in the model from Task 2D after scaling the variables and checking for collinearity and multicollinearity.

RESULTS

Task 3A&B: Sampling Texas tortoises and genome sequencing

A total of 10 Texas tortoises were sequenced. Given the representation of Texas tortoises from the study sites from Task 1 in McCracken et al. 2025, we sampled tortoises from Live Oak, Frio, Goliad, Uvalde, Duval, and Kleberg counties to increase the number of tortoises without NSD (Figure 13). Of these additional 10, 20% had signs of NSD. The sequence data for all ten tortoises are available on Sequence Read Archive (SRA) on study SRP514820. The specific accession numbers for these tortoises are listed in Table 2.

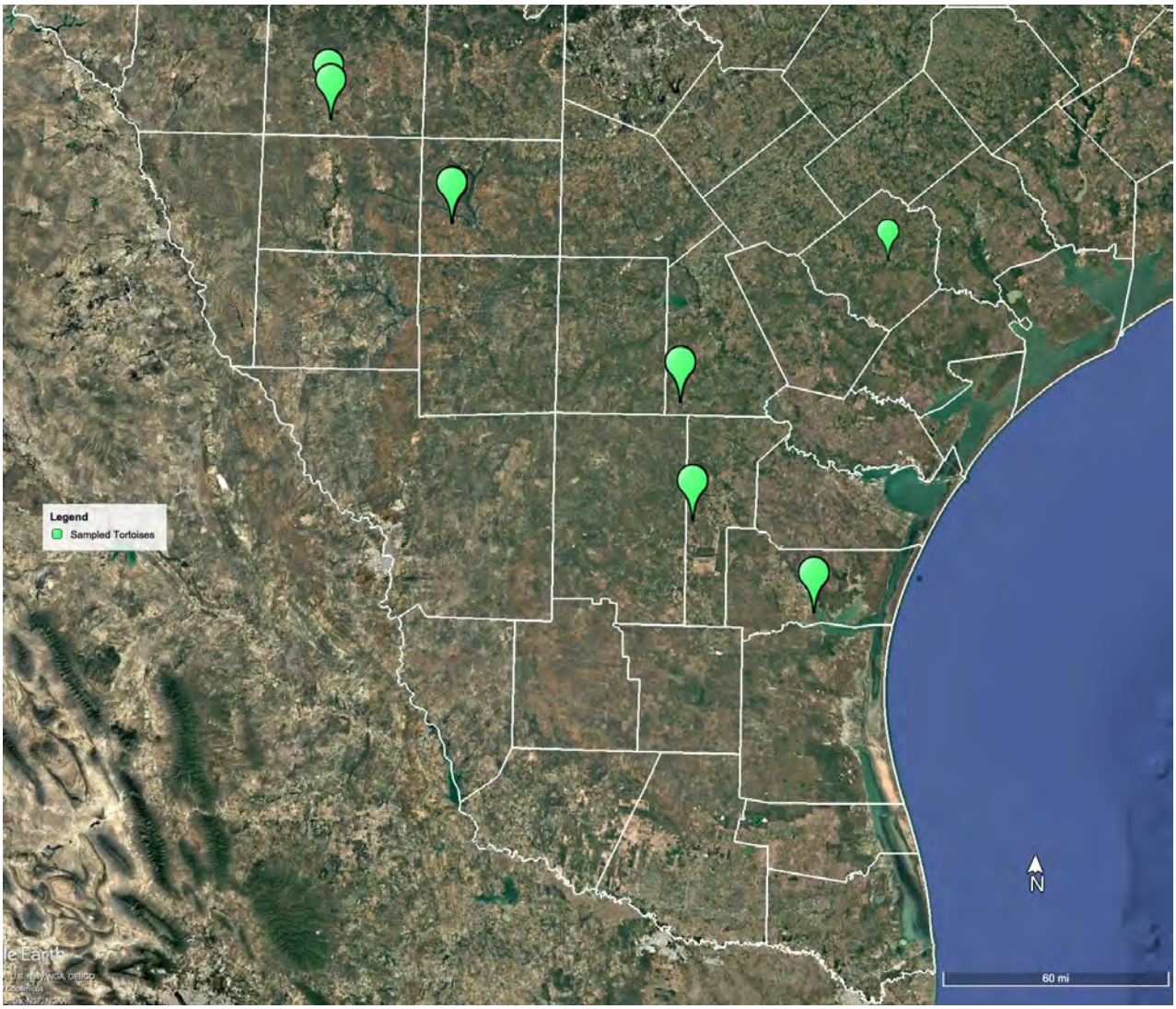


Figure 13. Map displaying approximate locations of the Texas tortoises sampled, represented by green dots.

Accession Number	Study	Sample Code
SRR33459452	SRP514820	JT087
SRR33459451	SRP514820	JT110
SRR33459450	SRP514820	JT111
SRR33459449	SRP514820	JT112
SRR33459448	SRP514820	JT113
SRR33459447	SRP514820	JT114
SRR33459446	SRP514820	JT115
SRR33459445	SRP514820	JT116
SRR33459444	SRP514820	JT117
SRR33459443	SRP514820	JT118

Table 2. List of accession numbers associated with Task 3B of this contract, uploaded onto SRA.

Task 3C: Compare genomes of Texas tortoises

With unpublished data from McCracken et al. 2025, a total of 48 Texas tortoises were compared. Based on the t-tests, there was no significant difference in observed heterozygosity or Wright's F based on NSD status (Table 3). When included on the global model from Task 2D, diversity metrics were the first dropped (Table 4).

Variable	t-statistic	Lower 95%	Upper 95%	p-value
Observed Heterozygosity	0.23	-0.01	0.02	0.82
Wright's F	-0.22	-0.04	0.05	0.82

Table 3. Summary statistics from Welch's t-test to compare individual diversity metrics based on NSD status.

Variable	Estimate	Standard Error	z-value	p-value
NSD Status ~ rainfall + sorghum + pasture + distance to tortoise + wright's f				
Rainfall	0.55	0.41	1.35	0.18
Sorghum	-0.57	0.41	-1.39	0.17
Pasture	-0.20	0.46	-0.43	0.67
Tortoise	-0.65	0.41	-1.59	0.11
Wright's F	-0.80	0.33	-0.24	0.81

Table 4. Summary statistics from binomial GLM with NSD status as response variable and rainfall, distance to sorghum, distance to pasture, distance to nearest tortoise, and Wright's F as response variables.

CHAPTER V: DISCUSSION

The most commonly isolated *Fusarium* spp. in this study was within the FIESC, but there were isolates across different species complexes. Isolates were also identified within FFSC, FSSC, and FOSC, potentially indicating that multiple species of *Fusarium* may lead to NSD. The initial etiological agent was identified as *Fusarium semitectum* (syn. = *Fusarium incarnatum*) (Rose et al. 2001); however, multiple fungi are often associated with one condition. Grain mold on sorghum can be caused by fungi in FIESC and FOSC (Prom et al. 2020). Additionally, stalk rot in sorghum is associated with nearly a dozen different species within the FFSC (Tesso et al. 2010; Klittich and Leslie 1992). In sea turtles, while sea turtle egg fusariosis (STEF) is linked to two fungi within the FSSC (Smyth et al. 2019; Sarmiento-Ramírez et al. 2014), representatives from FFSC and FOSC have been isolated from failed sea turtle eggs and nests (Gambino et al. 2020; Neves et al. 2015; Elshafie et al. 2007). Given that species from all the complexes represented have demonstrated the ability to degrade keratin (Preczeski et al. 2020; Kotwal and Sumbali 2016; Kannahi and Ancy 2012; Rose et al. 2001; Oyeka and Gugnani 1998), multiple species of *Fusarium* may be related to NSD. Further studies fulfilling Koch's postulates would be needed to confirm whether multiple fungi are responsible for NSD in Texas tortoises.

It is important to note that in only ~40% of cases, *Fusarium* spp. was successfully isolated from a tortoise exhibiting signs of NSD. This result could be in part due to the culturing methods. Malachite green agar (MGA) selects for the growth of *Fusarium* rather than common fungal contaminants (Alborch et al. 2010; Bragulat et al. 2004). In the initial trials, however, PCR from MGA samples was unsuccessful, so I elected to use PDA for the remaining cultures. This non-selective media (PDA) allowed for other non-target fungi (i.e., *Alternaria*, *Lasiodiplodia,* and *Trichoderma*) to grow, and these fungi may have competed with *Fusarium* (Sarrocco et al. 2019; Jensen et al. 2016). More rapid colonization by competitors (Sarrocco et al. 2019) may have created unfavorable conditions for the *Fusarium* to grow. However, other genera of fungi may also be involved in this condition or similar lesions in tortoises. Isolates putatively identified as *Aspergillus* spp. were also only isolated from NSD-positive sites, though at a lower rate and with one isolate (of 4) isolated from a tortoise demonstrating no signs of NSD. Species of *Aspergillus*, like *Fusarium*, can break down keratin (Alwakeel et al. 2021; Preczeski et al. 2020), though *Aspergillus* spp. are frequently noted to produce fewer keratinases than *Fusarium* (Alwakeel et al. 2021; Preczeski et al. 2020; Călin et al. 2017). *Aspergillus* have been cultured from failed sea turtle eggs and nests (Ambakesari et al. 2024; Candan 2018; Elshafie et al. 2007), though no inoculation trials have been conducted to confirm whether these fungi are related to nest failure. This is another area of research that requires further study to definitively confirm whether additional fungi are responsible for NSD lesions or whether lesions created by *Fusarium* facilitate the invasion of other keratinolytic fungi.

There was no indication that the *Fusarium* isolates were more related by collection location or by host. Modeling conferred a similar pattern where proximity to sorghum was only weakly associated with the probability of apparent NSD. Additionally, none of the other variables related to agriculture were correlated with the probability of NSD. *Fusarium* can be correlated with diseases in sorghum (Kent et al. 2022; Prom et al. 2020; Castor and Frederickson 1980), corn (Ortiz et al. 2015), and cotton (Diaz et al. 2021; Zhu et al. 2021) in Texas. However, hybrids of these crops are commonly bred for resistance to *Fusarium* (Yu et al. 2025; Tesso et al.

2010), which may have inhibited the ability to isolate these species from sorghum. To account for this, soil samples were also collected from each site. Although the next generation sequencing has not yet been conducted, gel electrophoresis of the *Fusarium*-specific primers (Karlsson et al. 2016) confirmed the presence of *Fusarium* at all sites, including the control. Sequencing is needed to confirm whether the same species of *Fusarium* associated with NSD were present in the soil, but spores of NSD causal agents are likely present throughout the range of the Texas tortoise. *Fusarium* spp. are widely distributed and have been obtained from atmospheric rainfall dust, air samples, and rainwater, suggesting long-distance dispersal (Harris and Pedersen 2011; Palmero et al. 2010). More likely, environmental conditions select where these *Fusarium* can impact tortoises, as appears to be the case with FSSC (Sarmiento-Ramírez et al. 2014). In most nesting beaches that were tested, FSSC was detected in the sand, but nesting mortality rates differed based on tidal inundation and soil composition (Sarmiento-Ramírez et al. 2014). Similarly, growth rates of *Fusarium* causal agents on crops varied based on temperature and soil pH (Panwar et al. 2016; Xu et al. 2008; Brennan et al. 2005; Rossi et al. 2001). Different local climatic factors can alter fungal growth rates and pathogenicity. Understanding the optimal environmental conditions under which NSD causal agents can grow and colonize Texas tortoises is critical information for predicting the spread of this disease.

Precipitation was the other variable weakly associated with apparent NSD. Higher amounts of precipitation can correspond with greater soil moisture, which can be critical for ascospore production and release in some *Fusarium* (Manstretta and Rossi 2015; Xu 2003). Precipitation alone, however, is not the only variable related to soil moisture important for ascospore production (Manstretta and Rossi 2015). The gradient of relative humidity across the sampled range also varies and, given how relative humidity correlates to with *Fusarium* growth (Panwar et al. 2016; Xu et al. 2008; Brennan et al. 2005; Doohan et al. 2003) and ascospore production (Manstretta and Rossi 2015; Xu 2003), may be an additional variable worth considering in its impact on creating suitable conditions for fungal growth. Another factor to explore in relation to the presence of apparent NSD on the landscape is the presence of other hosts on the landscape. Red-eared sliders (*Trachemys scripta*) have demonstrated that they can act as carriers of multiple species within the FSSC and have been proposed as a potential reservoir of STEF (Martínez-Ríos et al. 2022). In vitro, inoculating red-eared slider eggs with FSSC has led to high mortalities and low hatchling success (Martínez-Ríos et al. 2025), so it seems more likely that these turtles act as another host of FSSC. However, given that red-eared sliders can occur in the same habitats as Texas tortoises (Hellgren et al. 2000), the co-occurrence of these *Fusarium* pathogens in both hosts is worth exploring.

FUTURE RESEARCH RECOMMENDATIONS

The current literature available on NSD in Texas tortoises has focused exclusively on adults (Rose et al. 2001; Smith 2000). It was previously noted that all juveniles from an NSD-positive site had extensive keratin degradation on their plastron (Rose et al. 2001), but the impact of this degradation on juvenile development and survival is unknown. The keratin degradation likely weakens the integrity of the shell (Achrai and Wagner 2017). For juvenile tortoises that are susceptible to higher predation by a broader range of predators than adults (Rose and Judd 2014), a weakened shell could further increase the risk of predation to young and subsequently lower recruitment rates at sites with NSD. In loggerhead sea turtles, the same fungi related to STEF

were also isolated from skin and shell lesions of post-hatchlings (Greef-Laubscher and Jacobs 2022). Although the impacts of these lesions on post-hatchling turtles are unknown (Greef-Laubscher and Jacobs 2022), STEF has been linked to mass mortalities in sea turtle nests (Sarmiento-Ramírez et al. 2014; Sarmiento-Ramírez et al. 2010). As with sea turtles (Miller et al. 2003), female Texas tortoises will dig a chamber to lay their eggs when the soil is soft enough (Judd and Rose 1989). The contact or even complete coverage of eggs with soil where *Fusarium* is present could provide an opportunity for colonization of the egg (Hoh et al. 2020). Texas tortoise nests can be challenging to track (Hellgren et al. 2000); in fact, it has been decades since literature was published on wild tortoise nests (Judd and Rose 1989). Given the infrequent detection of nests and Texas tortoise eggs in the wild, the growth of fungus on eggs may have gone unnoticed. Studying the impacts of *Fusarium* spp. on Texas tortoise hatching and recruitment success would be vital to assess the risk these fungi pose to this species.

This research provides insight into where apparent NSD is most prevalent and brings into question whether multiple *Fusarium* spp. may be associated with this condition. This information is vital for targeting sites where future research on this disease can continue. With *Fusarium* pathogens having the ability to survive outside of their hosts as saprophytes (Banihashemi and Dezeeuw 1973), it is important to characterize which environmental conditions allow these fungi to thrive. Alongside calculating impacts on eggs and juveniles, these data can be used to better assess how and where these fungi are impacting Texas tortoises. Finally, individual inbreeding and observed heterozygosity were not predictors of disease status. Further examination of genes related to shell growth or immune function would be interesting to determine whether the fungus is exerting selective pressure on genes related to the shell.

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