POPULATION GENETICS OF THE BIG BEND SLIDER (*TRACHEMYS GAIGEAE GAIGEAE)* AND THE RED EARED SLIDER (*TRACHEMYS SCRIPTA ELEGANS)* IN THE CONTACT ZONE IN THE LOWER RIO GRANDE DRAINAGE OF TEXAS

by

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ABSTRACT

The red-eared slider (*Trachemys scripta elegans*) is well-known for its popularity in the pet trade. It is also known for its near cosmopolitan distribution, which is partly due to the release of these pet turtles. When introduced to a new area, non-native *T. s. elegans* can hybridize with other native *Trachemys* species. An example of this occurs between *T. s. elegans* and the Big Bend slider (*T. gaigeae gaigeae*) in western Texas. Recent research and trapping efforts have primarily focused on Big Bend National Park. Mitochondrial haplotypes unique to *T. g. gaigeae* have been observed in *T. s. elegans* inhabiting Rio Grande tributaries downstream of the park, which could indicate historical hybridization. This study sought to address these concerns by utilizing additional sampling within these areas. I used twenty polymorphic microsatellite loci and model-based clustering methods to detect hybrids. Out of the 120 turtles sampled, 7.5% were identified as hybrids using the program *Structure* v2.3.4, and 23.3% were identified as hybrids using NewHybrids v1.1. My results supported the findings of past research because hybridization was found between *T. g. gaigeae* and *T. s. elegans*. My results also supported the contention that morphology cannot be used to identify hybrids. Some of the backcrossed individuals were located in areas outside of the range of *T. g. gaigeae*. This may represent an ancestral polymorphism caused by previous gene flow between individuals in the Rio Grande, Pecos River, and Devils River.

I. INTRODUCTION

Rhymer and Simberloff (1996) define hybridization as the "interbreeding of individuals from what are believed to be genetically distinct populations regardless of the taxonomic status of such populations." It can occur when a new species is introduced to an area, as well as by the breakdown of reproductive isolation between native species (Rhymer and Simberloff 1996). Hybridization can have negative consequences on both hybrid offspring and parental populations. In some cases, hybrid offspring or their descendants, display fitness reduction known as outbreeding depression; however, hybrid vigor, in which hybrid offspring have increased fitness, is more often reported (Rhymer and Simberloff 1996). If hybrid offspring are capable of backcrossing with either of the parent populations, introgression of one genome into another is also possible (Rhymer and Simberloff 1996, Cureton et al. 2011).

Hybridization often poses a greater risk for populations that are small and/or isolated because they are more likely to be displaced by the non-native species (Huxel 1999). Difficulty finding potential mates of their own species may lead individuals to mate with those of another species. This problem escalates when the new species is present in large numbers because the likelihood of interbreeding increases. Even if hybrid offspring are sterile, reproductive efforts of the parent populations are wasted. This can especially harm populations that are few in numbers (Rhymer and Simberloff 1996, Cureton et al. 2011).

The red-eared slider, *Trachemys scripta elegans*, is native to the southeastern United States (Ernst and Lovich 2009). Ernst and Lovich (2009) refer to it as "the world's most widespread freshwater turtle" because it has been introduced to all

states within the United States except Alaska and all continents except Antarctica via the pet trade (Ernst and Lovich 2009). The red-eared slider was listed as one of "100 of the World's Worst Invasive Alien Species" (Lowe et al. 2000) and is known to compete with native turtles for basking sites (Spinks et al. 2003).

In addition to competing with native turtles, non-native *T. s. elegans* also hybridize with other native *Trachemys* species. Powell and Incháustegui (2009) reported hybridization between *T. s. elegans* and two native freshwater turtles of the Dominican Republic, *Trachemys decorata* and *Trachemys stejnegeri*. However, they did not state whether this observation was based on morphology or if hybrids were identified using genetic markers. While the presence of intermediate morphological characteristics might suggest that the two species are interbreeding, molecular techniques are required to confirm that there are hybrids within the population (Rhymer and Simberloff 1996). Parham et al. (2013) utilized nuDNA to find evidence of hybridization between *T. s. elegans* and *Trachemys stejnegeri* in Puerto Rico. They also noted introgression of the *T. scripta* genome into that of one *Trachemys decussata angusta* sampled in the Cayman Islands.

Intermediate forms thought to be hybrids of *T. s. elegans* and native *Trachemys taylori* have been reported in the Cuatro Ciénegas basin in Mexico (McGaugh 2012). McGaugh (2012) used microsatellite loci to determine if there was molecular evidence for hybridization; none was detected in the analysis. She suggested that hybridization could be a recent event and the lack of detected hybrids could be due to limited sampling of juvenile turtles. It was also suggested that the different courtship behavior of the two species could act as an isolation mechanism

(McGaugh 2012). Male *T. s. elegans* have long foreclaws that are used to stroke the face of the female during courtship (Jackson and Davis 1972, Ernst and Lovich 2009). Male *T. taylori* lack these foreclaws, and their courtship ritual consists of chasing a female and biting her (Davis and Jackson 1973).

The courtship behavior of male *T. g. gaigeae* also differs from that of *T. s. elegans*. Like *T. taylori*, male *T. g. gaigeae* lack long foreclaws. During courtship, a male will try to engage a female by swimming in front of her and bobbing his head (Stuart and Ward 2009). Despite this difference, molecular techniques confirmed hybridization between *T. s. elegans* and the Big Bend slider (*Trachemys gaigeae gaigeae*) in western Texas (Jackson 2010, Forstner et al. 2014).

The Big Bend slider, *T. g. gaigeae*, is a medium sized emydid turtle found in riparian habitats of the Rio Grande drainage system in Mexico, New Mexico, and Texas (Seidel et al. 1999, Stuart and Ward 2009). Although it was once thought to be a subspecies of *T. scripta*, it is now considered a unique species (Seidel 2002, Jackson et al. 2008). Lovich and Ennen (2013) listed *T. g. gaigeae* as one of the ten most poorly studied turtles and tortoises of the United States and Canada.

Currently, *T. g. gaigeae* is a species of conservation concern and was listed in 1996 as a species vulnerable to extinction by the International Union for Conservation of Nature and Natural Resources (IUCN) (Stuart and Ward 2009). Analysis of the sampling efforts of Forstner et al. (2014) in 1997 and 1998 estimated that there were approximately 7,500 *T. g. gaigeae* in the United States (with 4,500 in Texas, and 3,000 in New Mexico). The results of a later study, which included extensive sampling and mark recapture analyses within Big Bend National Park and Big Bend

State Ranch from 2005 to 2009, led to an estimated population size of 681 individuals for that region (Jackson 2010). Jackson (2010) also found two distinct populations of *T. g. gaigeae*. One population was located in New Mexico and the other was located in Texas. This supported the idea that *T. g. gaigeae* no longer inhabited most of its range between the Caballo reservoir in New Mexico and the confluence of the Rio Conchos in Presidio, Texas. Jackson (2010) recommended that the conservation and regulatory status of *T. g. gaigeae* should be elevated due to a low effective population size, low genetic diversity, and population structure. However, his study focused only on populations of *T. g. gaigeae* within the United States. An assessment of the Mexican population would be needed to change its status under global review (Jackson 2010). *Trachemys gaigeae gaigeae* faces several threats including habitat loss, fragmentation, and hybridization with its congener, *T. s. elegans* (Seidel et al. 1999, Stuart and Ward 2009).

The distributions of *T. s. elegans* and *T. g. gaigeae* were historically separated. In the Rio Grande, *T. g. gaigeae* ranged from Bosque del Apache National Wildlife Refuge in New Mexico to approximately the Brewster – Terrell county line in Texas. *Trachemys scripta elegans* was likely only native to the lower portions of the Rio Grande downstream of Big Bend National Park (Stuart and Ward 2009). Previously, native *T. s. elegans*, as well as hybrids between the two species, were found in locations within the range of *T. g. gaigeae*. In one case, a hybrid individual was captured 35 river miles upstream of the Brewster – Terrell county line in 1998. This individual displayed an intermediate phenotype, and hybrid status was confirmed using six microsatellite loci. This individual had alleles belonging to *T. g.*

gaigeae and *T. s. elegans* (Forstner et al. 2014). In 1998, another *T. s. elegans* was captured within Big Bend National Park at Rio Grande Village, but was thought to be a released pet and not the result of a native *T. s. elegans* migrating upstream (Forstner et al. 2014). Such releases of pet turtles have become a reoccurring problem in recent years. Non-native *T. s. elegans*, introduced into the Rio Grande in the form of unwanted pets, are problematic because they increase the probability of hybridization (Jackson 2010).

Previous work utilized microsatellites to confirm the presence of *T. g. gaigeae* and *T. s. elegans* hybrids in Big Bend National Park (Jackson 2010). The study found hybridization between these two species and identified four clusters of potential parent populations for *T. s. elegans*: 1) those found in eastern and central Texas, 2) those found in southeast and northeast Texas as well as Louisiana, Georgia, and Florida, 3) those native to the southern Rio Grande, and 4) those with questionable ancestry.

In addition to microsatellites, mitochondrial DNA has been used to assess hybridization in western Texas. Jackson's (2010) analysis of mitochondrial DNA in *Trachemys* throughout Texas found mitochondrial haplotypes unique to *T. g. gaigeae* in *T. s. elegans* inhabiting Rio Grande tributaries. However, these rivers are not part of the current range of *T. g. gaigeae*. It is possible that this ancestral polymorphism is due to historical hybridization between the two species (Jackson 2010).

Obviously, downstream contamination by introduced red-eared sliders is a concern within the Rio Grande itself. Very little is known about the presence of *T. s. elegans* within the Rio Grande at El Paso, Texas, because few records of *T. s. elegans*

within El Paso exist. One *T. s. elegans*, thought to be an escaped captive, was found in El Paso County in 1965 (UTEP Herpetology Collection). Additional *T. s. elegans* were found in El Paso County in 2008 and 2009 (MRJ Forstner Frozen Tissue Collection). Because the Rio Grande is subject to extreme flooding events, it would be beneficial to determine if *T. s. elegans* inhabiting El Paso urban areas can disperse downstream where they might come into contact with populations of *T. g. gaigeae*. Huxel (1999) found that the rate of displacement of a native species was largely influenced by amount of immigration and level of fitness of the non-native species. Increased immigration of *T. s. elegans* from upstream could have an increasing impact on the displacement rate of *T. g. gaigeae* by direct competition, introgression, or other means.

The first objective of my study was to conduct a current analysis of the extent of hybridization downstream of Big Bend National Park near the native range of *T. s. elegans*. The second objective was to examine the prevalence of hybrids found in tributaries of the Rio Grande because they may be the result of ancestral polymorphism.

II. METHODS

Study Site

To address the first objective, turtles were obtained from two areas of interest downstream of Big Bend National Park. The first, Black Gap Wildlife Management Area, was located directly downstream of Big Bend National Park. The second, the Lower Canyons of the Rio Grande, was located downstream of Black Gap Wildlife Management Area and continued to just beyond the Brewster – Terrell county line. This is the edge of the downstream range of *T. g. gaigeae.* To address the second objective, turtles were obtained from three main areas along Rio Grande tributaries. These areas included The Nature Conservancy's Independence Creak Preserve (Oasis Ranch) along the Pecos River, The Nature Conservancy's Dolan Fall Preserve along the Devils River, and the city of Del Rio along San Felipe Creek. These tributaries were outside the range of *T. g. gaigeae*.

Tissue Collection

I used 113 blood or tissue samples previously obtained from *Trachemys gaigeae gaigeae*, *Trachemys scripta elegans*, and suspected hybrids and maintained in the MRJ Forstner Frozen Tissue Collection housed at Texas State University. Because my study focused on areas that are downstream of Big Bend National Park, the majority of samples were from turtles captured in Black Gap Wildlife Management Area (2 *T. s. elegans*, 5 suspected hybrids), the Lower Canyons (2 *T. s. elegans*, 10 *T. g. gaigeae*, 5 suspected hybrids), Terrell County (10 *T. s. elegans*, 1 suspected hybrid), and Val Verde County (33 *T. s. elegans*). Additional samples came from New Mexico (4 *T. s. elegans*, 3 *T. g. gaigeae*), El Paso County (3 *T. s. elegans*,

2 *T. g. gaigeae*), Hudspeth County (10 *T. g. gaigeae*), Presidio County (2 *T. s. elegans*, 6 *T. g. gaigeae*), and Big Bend National Park (3 *T. s. elegans*, 10 *T. g. gaigeae*, 2 suspected hybrids) (Figure 1).

Tissue and blood collection from these turtles occurred between 1998 and 2010 with most of the sampling events occurring in late April through July. This is known to be to the most active season for *T. g. gaigeae* (Jackson 2010). Jackson (2010) identified fourteen of these individuals (MRJ Forstner Frozen Tissue Collection numbers: 2040, 2041, 2316, 5872, 5873, 6186, 18854, 18866, 19178, 19292, 20734, 21513, 27603, 27605) as hybrids using microsatellite markers. He also identified fourteen of these individuals (MRJ Forstner Frozen Tissue Collection numbers: 9712, 9803, 9886, 18859, 18898, 18900, 18929, 19061, 19089, 19208, 19394, 19570, 21512, 21514) with conflicting identification depending on whether morphological or microsatellite sources were used.

An additional seven samples were collected from The Nature Conservancy's Independence Creek Preserve (Oasis Ranch) (1 *T. s. elegans*) in Terrell County and The Nature Conservancy's Dolan Falls Preserve along the Devils River and San Felipe Creek in Del Rio, Texas (6 *T. s. elegans*) in Val Verde County. These samples were collected from 2013 – 2014.

Sampling Protocol

In areas of suitable habitat, hoop nets were baited with sardines and deployed. Suitable habitat for *T. g. gaigeae* included deeper pools adjacent to riffles in the Rio Grande (Forstner et al. 2014). Sardines used to bait the nets were stored in non-consumable containers. Each hoop net was secured to vegetation to prevent it from

floating away and contained a flotation device to prevent total submersion of the hoop net and any turtles from drowning. If water clarity allowed, hand capture of turtles was attempted.

All turtles captured were assigned to *T. g. gaigeae*, *T. s. elegans*, or a suspected hybrid group based on morphology (Figure 2). Morphological characteristics (sex, carapace length and width, plastron length and width, body depth, and weight) were recorded and photographs were taken for all turtles. Less than 0.1 ml of blood was collected from the femoral vein using a 25-gauge needle and 1.0 ml syringe. Blood samples were stored in blood storage buffer (100mM Tris pH 8.0, 100mM Na2 EDTA, 10mMNaCl and 1%SDA), and were kept at -80°C for long-term storage. Turtles were marked by shell-notching and/or passive integrated transponder (PIT) tags to identify future recaptures. Turtles were released at their capture site after this information was collected. These procedures were approved by the Texas State Institutional Animal Care and Use Committee (Protocol# 0417_0513_08).

Molecular Analysis

DNA was extracted from blood and tissue samples using the Qiagen DNeasy Blood and Tissue kit or using a Wizard SV® SV 96 Genomic DNA Purification System with a Biomek® 3000 Laboratory Automation Workstation. Gel electrophoresis was used to confirm the success of DNA extraction.

I amplified twenty-three polymorphic microsatellite loci in order to detect hybrids (Table 1). Thirteen of these microsatellite loci were previously used by Jackson (2010) to identify hybrids between *T. g. gaigeae* and *T. s. elegans*. Nine of

the thirteen (GmuA19, GmuB08, GmuB21, GmuD28, GmuD55, GmuD70, GmuD87, GmuD93, and GmuD121) were developed by King and Julian (2004). Three loci (MT3, Tufu-2, Pseud 4-128, and Pseud 225-2) were developed by Forstner et al (2014). One locus (MT3) was developed by Forstner and Davis (unpublished data). The remaining ten microsatellite loci (Tsc108, Tsc169, Tsc241, Tsc243, Tsc252, Tsc260, Tsc263, Tsc299, Tsc302, Tsc323) were developed by Simison et al. (2013) to aid studying the population genetics of native and invasive *T. s. elegans* and also identify *T. s. elegans* within populations where it coexists with conspecifics. Each forward primer was designed to include a M13 (-21) tail (5'-TGT AAA ACG ACG GCC AGT-3') on the 5' end. Universal M13 (-21) primers were ordered with one of four florescent dyes (NED-TGT AAA ACG ACG GCC AGT-3', PET- TGT AAA ACG ACG GCC AGT-3', FAM- TGT AAA ACG ACG GCC AGT-3', or VIC- TGT AAA ACG ACG GCC AGT-3'). This follows Schuelke's (2000) methods for fluorescent labeling of PCR fragments.

Each 25ul PCR reaction contained: 0.00255mg bovine serum albumen, 1x Taq Buffer (Genscript), 0 - 0.5 mM additional $MgCl_2$, 0.1 mM dNTP's, 0.04uM forward primer, 0.08 uM reverse primer, 0.08 uM universal M13 (-21) primer, 0.5 units Taq (Genscript), and 1 ul extracted DNA. Thermal cycling consisted of an initial denaturation at 94°C for 2 minutes followed by 30 cycles consisting of a denaturation period of 45 seconds at 94°C, annealing period of 45 seconds at 54°C - 60°C, and an extension period of 1 minute 30 seconds at 72°C. This was followed by 8 additional cycles consisting of a denaturation period of 30 seconds at 94°C, an annealing period of 45 seconds at 53°C, and an extension period of 45 seconds at 94°C. These

additional cycles allowed the universal M13 (-21) primer to anneal to the PCR product. A final extension period of 10 minutes at 72°C ended the reaction.

I genotyped the labeled amplicons using an ABI 3500 XL Genetic Analyzer, and identified peaks using the GeneMapper Software v4.1 by Applied Biosystems. The program CREATE v1.37 (Coombs et al. 2008) was used to ensure that all data was properly formatted for analysis. MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) was used to detect any genotyping errors.

Programs *Structure* v2.3.4 (Pritchard et al. 2000) and NewHybrids v1.1 (Anderson and Thompson 2002) were used to identify hybrid individuals. The admixture model in *Structure* v2.3.4 was used to test the number of populations (K) at multiple values (1 through 8). For each value of K, there were five independent replicates. Each replicate consisted of a burn in of 10,000 followed by 100,000 MCMC repetitions. Structure Harvester v0.6.94 (Earl and VonHoldt 2012) was use to determine the best K value. This was done by calculating ΔK following the methods of Evanno et al. (2005). Individuals were assigned to a population if they had 80% or greater assignment probability for that population. All others were considered hybrids.

NewHybrids v1.1 was used to determine the probability of an individual belonging to one of six genotype frequency classes (species 1, species 2, F1 hybrid, F2 hybrid, backcross with species 1 or backcross with species 2). The default proportions for each genotype frequency class were used. A Jeffrey's prior was used for mixing proportions (π) and allele frequencies (θ). No other prior information was used. The simulation was run with a burn in of 250,000 followed by 1,000,000

MCMC repetitions. Individuals were assigned to a one of the genotype frequency classes if they had 80% or greater average assignment probability for that class. All others were considered hybrids.

III. RESULTS

Three of the twenty-three loci (Tsc243, Tsc263, and MT3) did not amplify consistently and were excluded from further analyses. One of the loci (MT3) amplified under normal PCR conditions, but did not amplify when the M13 (-21) florescent marker was included in the reaction. When all of the samples were analyzed in MICRO-CHECKER v2.2.3, excess homozygotes were reported at all loci. This number was reduced when the samples were divided by species. Five loci displayed excess homozygotes in *T. g. gaigeae* (Gmu A19, GmuB08, GmuB21, GmuD28, Tsc260) while fifteen loci continued to display excess homozygotes in *T. s. elegans* (GmuA19, GmuB08, GmuB21, GmuD28, GmuD55, GmuD70, GmuD87, GmuD93, GmuD121, Tufu2, Pseud 4-128, Tsc169, Tsc299, Tsc302, Tsc323). This was likely due to the Wahlund Effect, which states that excess homozygotes are likely to be detected if several small local populations are treated like one large population (Wahlund 1927, Sinnock 1975).

In *Structure* v2.3.4, the number of populations (K) was estimated to be two, with each population representing one of the two species (Figure 3). Out of the 120 turtles sampled, 92.5% were assigned to one of the populations (43 individuals to *T. g. gaigeae* and 68 individuals to *T. s. elegans*). Several of these individuals had conflicting morphological and molecular identification. Two individuals identified as *T. g. gaigeae* in the field were identified as *T. s. elegans*. Three individuals suspected to be hybrids were identified as *T. s. elegans* and six were identified as *T. g. gaigeae*. The remaining 7.5% were identified as hybrids. Within the hybrid individuals, four individuals were identified as suspected hybrids, two as *T. g. gaigeae*, and three as *T.*

s. elegans based on morphology (Table 2). The hybrid individuals were found in areas downstream of Big Bend National Park (24% of individuals captured in the Lower Canyons of the Rio Grande and 29% of individuals captured within the river boundaries of Black Gap Wildlife Management Area) or in areas within the range of *T. g. gaigeae* that had public access (100% of individuals captured in Lajitas, Texas and 7% of individuals captured in Big Bend National Park) (Figure 4).

Of the turtles sampled, 76.7% were assigned to one of the species using NewHybrids v1.1 (42 individuals to *T. g. gaigeae* and 50 individuals to *T. s. elegans*) (Figure 4). One individual was identified as *T. g. gaigeae* in the field, but was later identified as *T. s. elegans* using NewHybrids v1.1. This individual was also identified as *T. s. elegans* in *Structure* v2.3.4. Three individuals suspected to be hybrids were identified as *T. s. elegans* and six were identified as *T. g. gaigeae*. The remaining 23.3% were identified as hybrids. Within the hybrid individuals, four individuals were identified as suspected hybrids, four as *T. g. gaigeae*, and twenty as *T. s. elegans* based on morphology. Sixteen of these individuals were identified as the result of backcrossing. No individuals were identified as F1 or F2 hybrids (Table 2). The hybrid individuals were found in areas downstream of Big Bend National Park (47% of individuals captured in the Lower Canyons of the Rio Grande and 29% of individuals captured within the river boundaries of Black Gap Wildlife Management Area) or in areas within the range of *T. g. gaigeae* that had public access (14% of individuals captured in New Mexico, 40% of individuals captured in El Paso, Texas, 100% of individuals captured in Lajitas, Texas, and 20% of individuals captured in Big Bend National Park). Hybrid individuals were also found in tributaries of the Rio

Grande (50% of individuals captured in Oasis Ranch and 14% of individuals captured in Del Rio, Texas) (Figure 4).

IV. DISCUSSION

Hybrid individuals were identified with *Structure* v2.3.4 and NewHybrids v1.1. NewHybrids v1.1 identified more individuals as hybrids compared to *Structure* v2.3.4. All individuals that were considered hybrids in *Structure* v2.3.4 were also considered hybrids in NewHybrids v1.1. The increase in the number of hybrid individuals detected by NewHybrids v1.1 may be explained by the fact that NewHybrids v1.1 incorporates expected genotype frequency information into its analysis while *Structure* v2.3.4 does not (Anderson 2009). This information includes the proportion of alleles from each species that a F1 hybrid, F2 hybrid, or backcrossed individual would be expected to have.

Most of the hybrids were found in areas downstream of Big Bend National Park or in areas accessible to the public. No individuals were labeled as F1 or F2 hybrids according to NewHybrids v1.1 despite the presence of backcrossed individuals. In NewHybrids v1.1, a backcrossed individual was the result of an individual that was purely one species mating with an individual that was an F1 hybrid. Backcrossing can be detrimental to the parent populations as it can lead to introgression of one genome into another (Rhymer and Simberloff 1996, Cureton et al. 2011). Some of the backcrossed individuals were located in areas outside of the range of *T. g. gaigeae*. It is possible that hybrid turtles could have been recently introduced to these areas. However, Jackson (2010) found that hybrid individuals in these area often had a mitochondrial haplotype identifying them as *T. g. gaigeae*. He concluded this to be an ancestral polymorphism caused by previous gene flow between individuals in the Rio Grande, Pecos River, and Devils River.

My results support the findings of past research because hybridization was found between *T. g. gaigeae* and *T. s. elegans*. They also support the idea that morphology cannot identify hybrids. The number of populations (K) for my *Structure* v2.3.4 analysis was two, representing one population containing *T. g. gaigeae* and one population containing *T. s. elegans,* as was Jackson's (2010). Jackson's NewHybrids v1.1 analysis also identified more hybrid individuals than the *Structure* analysis, and all hybrids identified by *Structure* where also identified by NewHybrids v1.1 as in this study. However, in this study, 23.3% of individuals were identified as hybrids using NewHybrids v1.1 while 8.3% of individuals were identified as hybrids using NewHybrids v1.1 in Jackson's study.

There were some discrepancies when I compared the results of my *Structure* v2.3.4 and NewHybrids v1.1 analyses to those of Jackson (2010) for the same individuals. Forty-four individuals were used in this study and Jackson's study. Only fifteen of these individuals (34%) had similar assignments in *Structure* v2.3.4 and NewHybrids v1.1. There were several individuals that were identified in the field as *T. g. gaigeae*, but Jackson's analysis identified them as *T. s. elegans* (MRJ Forstner Frozen Tissue Collection numbers: 18859, 18900, 18929, 19061, 19089, 19394, 19570, 21512, 21514) or hybrids (MRJ Forstner Frozen Tissue Collection numbers: 18866 19202, 21513) molecularly. My *Structure* v2.3.4 and NewHybrids v1.1 analyses identified all of these individuals as *T. g. gaigeae*. Other individuals were identified in the field as *T. s. elegans*, but Jackson's analysis identified them as *T. g. gaigeae* (MRJ Forstner Frozen Tissue Collection numbers: 9712, 9886) or hybrid

(MRJ Forstner Frozen Tissue Collection numbers: 5872, 5873, 27603, 27605). These individuals were identified as *T. s. elegans* in this study.

While the two studies were similar, there were several key differences that may explain the disparities in the results. This study utilized twenty microsatellite loci, while Jackson used thirteen. However, Jackson had a larger sample size (192 individuals compared to 120 individuals in this study). The composition of the individuals used in each study varied as well. Jackson's samples consisted primarily of *T. g. gaigeae* (131 *T. g. gaigeae*, 56 *T. s. elegans*, and 6 suspected hybrids) while this study utilized more samples from *T. s. elegans*. NewHybrids v1.1 and *Structure* v2.3.4 are Bayesian methods for generating assignment probabilities. Since they generate the assignment probability of an individual based on the observed data, it is likely that the differences in the datasets are leading to these results.

Areas of Future Study

More research is needed to fully understand the impact hybridization is having on these turtles. *Trachemys gaigeae gaigeae* has been listed as one of the ten most poorly studied turtles and tortoises of the United States and Canada (Lovich and Ennen 2013). Very little published research has focused on the Mexican subspecies, *T. g. hartwegi*. It is unknown if these turtles are facing the same threats as *T. g. gaigeae*. More information on the status of this subspecies is needed to update the status of *T. gaigeae*.

Recent research and trapping efforts have primarily focused on turtles found within Brewster County, particularly those inhabiting the region near Big Bend National Park. Even though hybridization has been known to occur in the Lower

Canyons of the Rio Grande downstream of Big Bend National Park, it has been over fifteen years since turtles inhabiting this area have been sampled and analyzed to determine the extent of hybridization. Thus, updating the prior work with new samples and new, higher resolution genetic markers should be a primary goal of any future investigation.

Future research should also continue to address the presence of *T. s. elegans* in El Paso, TX as very little is known. It would be beneficial to determine if *T. s. elegans* inhabiting El Paso urban areas can disperse downstream where they could come into contact with populations of *T. g. gaigeae* as this could could have an increasingly negative effect on the rate of species displacement in *T. g. gaigeae*. It is also important to note that hybridization occurred in El Paso. However, only five individuals from this area were included in this study. More intensive sampling is needed to determine the extent of hybridization in this area.

Research Implications

Trachemys scripta elegans have been introduced to all states within the United States except Alaska and all continents except Antarctica via the pet trade (Ernst and Lovich 2009). Some of these areas are inhabited by other *Trachemys* species. In Brazil, *T. s. elegans* have been found in areas of habitat similar to that used by *T. dorbigni* (Ferronato et al. 2009, Bujes 2011). In Argentina, a single *T. s. elegans* has also been found in an area inhabited by *T. dorbigni*, which is considered endangered there (Alcalde et al. 2012).

Hybridization between *T. s. elegans* and two native freshwater turtles of the Dominican Republic, *Trachemys decorata* and *Trachemys stejnegeri*, has also been

reported, but it is unknown if this has been verified molecularly (Powell and Incháustegui 2009). Parham et al. (2013) found evidence of hybridization between *T. s. elegans* and *Trachemys stejnegeri* in Puerto Rico using nuDNA, and noted introgression of the *T. scripta* genome into that of one *Trachemys decussata angusta* sampled in the Cayman Islands. While *T. stejnegeri* is considered to be a lower risk, *T. decorata* is considered vulnerable by the IUCN (Tortoise & Freshwater Turtle Specialist Group 1996*a*, *b*). The implications of this study and future research are important not just to *T. g. gaigeae,* but to other rare and endangered members of the genus *Trachemys* as well.

Table 1. Microsatellite loci used to detect hybrids of *Trachemys gaigeae gaigeae* and *Trachemys scripta elegans*. Each forward primer included a M13 (-21) tail (5'-TGT AAA ACG ACG GCC AGT-3') on its 5' end. Gray samples represent those that did not amplify consistently.

Locus	Primer Sequence (5' - 3')	Repeat Motif	Observed Size	Reference
GmuA19	F:TAA GAG ACA GAT GCT CAG CAA G	$(GA)_7(GT)_{14}$	134 - 162	King and Julian (2004)
	R: GTA CAT AAC ACG CAC CCA ATG			
GmuB08	F: CTC TGA GAC CCT TAT TCA CGT C	$(TAC)_{10}$	231 - 279	King and Julian (2004)
	R: AGC CTT TGT CTG TAA GCT GTT C			
GmuB21	F: CTA GTT CGA AAC AGG ACC GTT G	$(TAC)_{10}$	226 - 274	King and Julian (2004)
	R: CCA CAC GAC AGT TTG ATG TCA G			
GmuD28	F: AGC TGT TTG TCA TCA TAC ACT CTC	$(ATCT)_{15}$	201 - 265	King and Julian (2004)
	R: TGG CCC TCA TGT TTT ATA AGT G			
GmuD55	F: GTG ATA CTC TGC AAC CCA TCC	$(ATCT)_{10}$	179 - 231	King and Julian (2004)
	R: TTG CAT TCA GAA TAT CCA TCA G			
GmuD70	F: AGT GTA GTC ATG GCA TAG AGA GG	$(ATCT)_8$	184 - 318	King and Julian (2004)
	R: ATC AAA TTC TTC CAA CCC TAC C			
GmuD87	F: AAA CCC TAA GAC ATC AGA CAG G	$(ATCT)_{22}$	224 - 288	King and Julian (2004)
	R: CAA ATC CAG TAC CCA GAA AGT C			
GmuD93	F: AGA CTC TCT TGA CCA GAT TTT CTC	$(ATCT)_{18}$	140 - 216	King and Julian (2004)
	R: TCT GCC TTC TAT CAC TCT CCT G			
GmuD121	F: GGC AAA TAT CCA ATA GAA ATC C	$(ATCT)_8$	149 - 193	King and Julian (2004)
	R: CAA CTT CCT CGT GGG TTC AG			
MT3	F: GCT GCA CAG AGT TAC TTG GCA AG		n/a	Forstner and Davis.
	R: ACC CAT CCA TTC TGA CAA TAG CTC			(Unpublished Data)
Tufu-2	F: TGC TCC TCA TTA TGG TAC AGG GTG		180 - 216	Forstner et al (2014)
	R: TCT GCC TCT CAC ACA CAA ACT CAG			
Pseud 4-128	F: GCA AGG CTG CAC AAA CTC TC		194 - 254	Forstner et al (2014)
	R: GCA GGT GTC CAC ATT GAC			
Pseud 225-2	F: TCC TCT ATT CAA CACA CC GAC CA		122 - 128	Forstner et al (2014)
	R: CCG CAG CAT ACT AAT TGA CTT TG			
Tsc108	F: CGC AGT CAA AAC ACC TTC AG	$(TAGA)_8$	222 - 290	Simison et al (2013)
	R: TTC ACC TCC CCA GAT CTC AC			
Tsc169	F: TAA AAT GGG CCT CAA CAA GG	$(TAGA)_{10}$	227 - 279	Simison et al (2013)
	R: GGA TTG TTT GGT CAA AGA AGT TG			
Tsc241	F: GGT TTT TCT CCA TCC CGA AT	$(TATC)_7$	204 - 228	Simison et al (2013)
	R: TTC ATT TGA AAG GTT AGC TCG T			
Tsc243	F: GCA AAA CCT GGA GAT TTT CAA	$(ATAG)_{20}$	n/a	Simison et al (2013)
	R: TTT CGA TGG AAA ATG GCT TT			
Tsc252	F: CCA TAC ACC CTC TGA CAG CA	$(ATAG)_8$	206 - 250	Simison et al (2013)
	R: TTC CCA AGA CAA GAA ACA CCT T			
Tsc260	F: TGC AAA TGG AGT TGC AAG A	$(ATCT)_{16}$	172 - 228	Simison et al (2013)
	R: TCC ATT TGA ACC TGG GAG AA			
Tsc263	F: TGT GCA CGG GAG TTG TAT G	$(GATA)_{10}$	n/a	Simison et al (2013)
	R: TTC TAT TTG CCA AAA ATT GCA T			
Tsc299	F: CCA TGT GCC ATC TGT CTA CCT	$(TATC)_{17}$	262 - 320	Simison et al (2013)
	R: GAT CAA GGG ATG AGG GTC AA			
Tsc302	F: ACT GGC CAG CAG GAG TAA TG	$(TAGA)_7$	178 - 290	Simison et al (2013)
	R: TGG GGC ACA AAC TAC TAG GG			
Tsc323	F: TGT AAA ATT GAT TAG GAC CTC TCT GA	$(TATC)_{14}$	212 - 260	Simison et al (2013)
	R: TGC AAT CTA TCA CAT GAC TGC AT			

Table 2. Individuals recognized as hybrids of *Trachemys gaigeae gaigeae* and *Trachemys scripta elegans* or individuals with conflicting morphological and molecular identification. In *Structure* v2.3.4 individuals were considered hybrids if their highest assignment probability was less than 0.8. In NewHybrids v1.1, individuals were considered hybrids if they had 0.8 or greater assignment probability for one of the hybrid classes (FI, F2, Backcross with Species 1, Backcross with Species 2) or if their highest assignment probability was less than 0.8. *Trachemys gaigeae gaigeae* is represented by *T. g. g.* and *Trachemys scripta elegans* is represented by *T. s. e*.

ID	Locality	Field ID	*Structure* v2.3.4 ID	NewHybrids v1.1 ID
406	Lower Canyons, TX	Hybrid	*T. s. e*	*T. s. e*
2014	Lower Canyons, TX	Hybrid	*T. g. g.*	*T. g. g.*
2015	Lower Canyons, TX	Hybrid	*T. g. g.*	*T. g. g.*
2018	Lower Canyons, TX	*T. g. g.*	*T. s. e*	Hybrid
2019	Lower Canyons, TX	*T. s. e*	*T. s. e*	Hybrid
2023	Lower Canyons, TX	*T. g. g.*	Hybrid	Backcross w/ *T. g. g.*
2024	Lower Canyons, TX	*T. g. g.*	*T. g. g.*	Backcross w/ *T. g. g.*
2025	Lower Canyons, TX	*T. g. g.*	Hybrid	Backcross w/ *T. g. g.*
2034	Lower Canyons, TX	Hybrid	Hybrid	Backcross w/ *T. s. e.*
2039	Lower Canyons, TX	Hybrid	*T. s. e*	*T. s. e*
2040	Lower Canyons, TX	Hybrid	Hybrid	Backcross w/ *T. s. e.*
2041	Lower Canyons, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
2316	Langtry, TX	*T. s. e*	*T. s. e*	Hybrid
6186	Oasis Ranch, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
8320	Oasis Ranch, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
8321	Oasis Ranch, TX	*T. s. e*	*T. s. e*	Hybrid
9803	Oasis Ranch, TX	*T. s. e*	*T. s. e*	Hybrid
18854	Big Bend National Park, TX	Hybrid	Hybrid	Backcross w/ *T. g. g.*
18898	Big Bend National Park, TX	Hybrid	*T. g. g.*	*T. g. g.*
19178	Big Bend National Park, TX	*T. s. e*	*T. s. e*	Hybrid
19208	Big Bend National Park, TX	*T. g. g.*	*T. s. e*	*T. s. e*
20519	Lajitas, TX	*T. s. e*	Hybrid	Backcross w/ *T. s. e.*
20734	Lajitas, TX	*T. s. e*	Hybrid	Backcross w/ *T. s. e.*
26859	Bosque Del Apache NWR, NM	*T. s. e*	*T. s. e*	Hybrid
26917	Del Rio, TX	*T. s. e*	*T. s. e*	Hybrid
26926	Del Rio, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
27352	El Paso, TX	*T. s. e*	*T. s. e*	Hybrid
27353	El Paso, TX	*T. s. e*	*T. s. e*	Hybrid
30343	Black Gap WMA, TX	Hybrid	Hybrid	Backcross w/ *T. s. e.*
30364	Black Gap WMA, TX	Hybrid	*T. s. e*	*T. s. e*
30386	Black Gap WMA, TX	Hybrid	*T. g. g.*	*T. g. g.*
30387	Black Gap WMA, TX	Hybrid	*T. g. g.*	*T. g. g.*
30388	Black Gap WMA, TX	Hybrid	*T. g. g.*	*T. g. g.*
30390	Black Gap WMA, TX	*T. s. e*	Hybrid	Backcross w/ *T. s. e.*
33081	Del Rio, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
33082	Del Rio, TX	*T. s. e*	*T. s. e*	Backcross w/ *T. s. e.*
33099	Del Rio, TX	*T. s. e*	*T. s. e*	Hybrid
35901	Oasis Ranch, TX	*T. s. e*	*T. s. e*	Hybrid

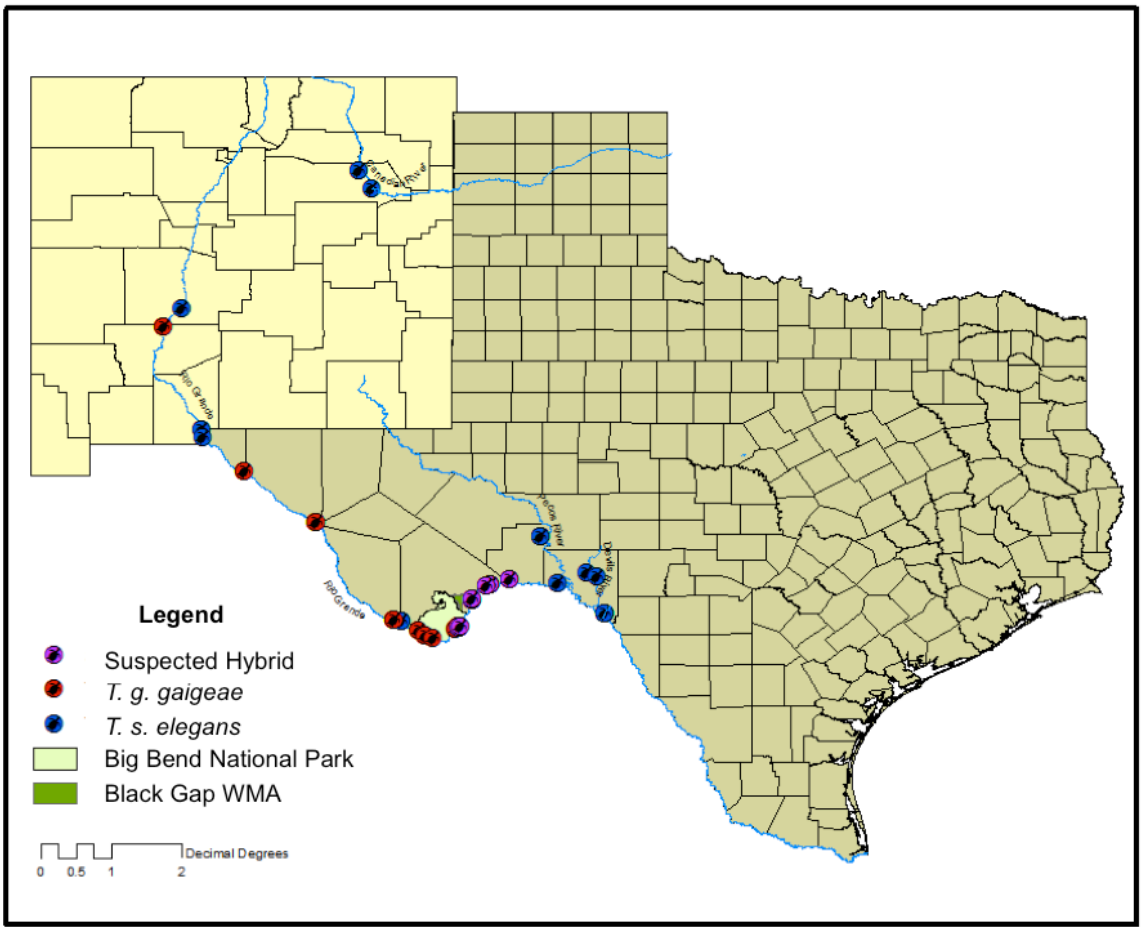

Figure 1. Locations of the 120 *Trachemys gaigeae gaigeae*, *Trachemys scripta elegans*, and suspected hybrids from Texas and New Mexico used in this study. This study focused on areas that are downstream of Big Bend National Park with the majority of samples coming from Black Gap Wildlife Management Area (2 *T. s. elegans*, 5 suspected hybrids), the Lower Canyons (2 *T. s. elegans*, 10 *T. g. gaigeae*, 5 suspected hybrids), Terrell County (11 *T. s. elegans*, 1 suspected hybrid), and Val Verde (39 *T. s. elegans*) County. Additional samples came from New Mexico (4 *T. s. elegans*, 3 *T. g. gaigeae*), El Paso County (3 *T. s. elegans*, 2 *T. g. gaigeae*), Hudspeth County (10 *T. g. gaigeae*), Presidio County (2 *T. s. elegans*, 6 *T. g. gaigeae*), and Big Bend National Park (3 *T. s. elegans*, 10 *T. g. gaigeae*, 2 suspected hybrids)

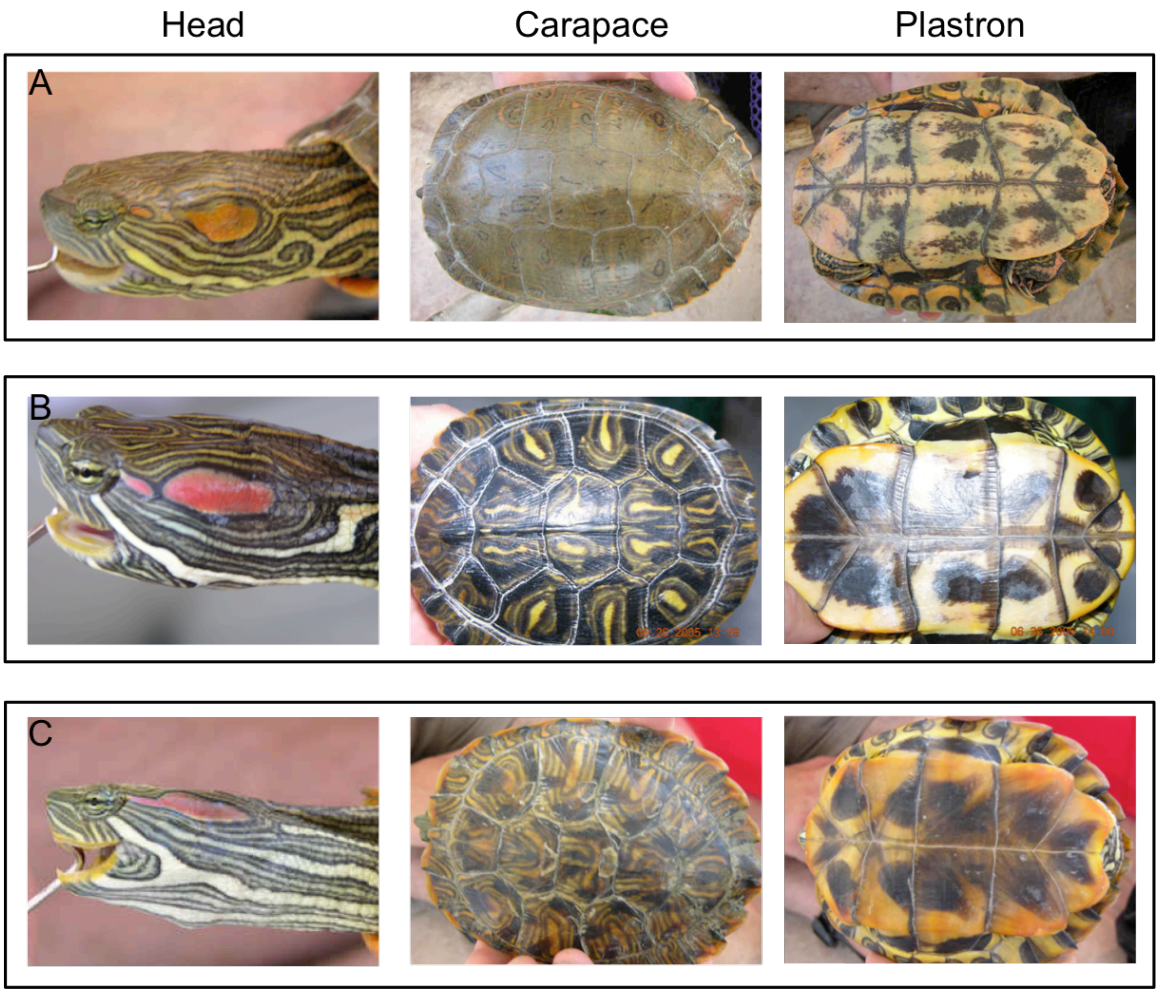

Figure 2. Morphological characteristics of *Trachemys gaigeae gaigeae* (A), *Trachemys scripta elegans* (B), and a suspected hybrid (C). *T. g. gaigeae* is differentiated from *T. s. elegans* by the presence of a black-bordered post orbital patch that does not touch the orbit. The carapace also has a pattern of light lines. Males lack long fore claws unlike *T. s. elegans*. *T. s. elegans* is distinguished by the presence of a long post orbital stripe that touches the orbit. Suspected hybrids are turtles displaying intermediate characteristics of *T. s. elegans* and *T. g. gaigeae*. While morphology alone is not a reliable method of identifying hybrids, in this case, the turtle was later identified by Jackson (2010) as a hybrid using microsatellites.

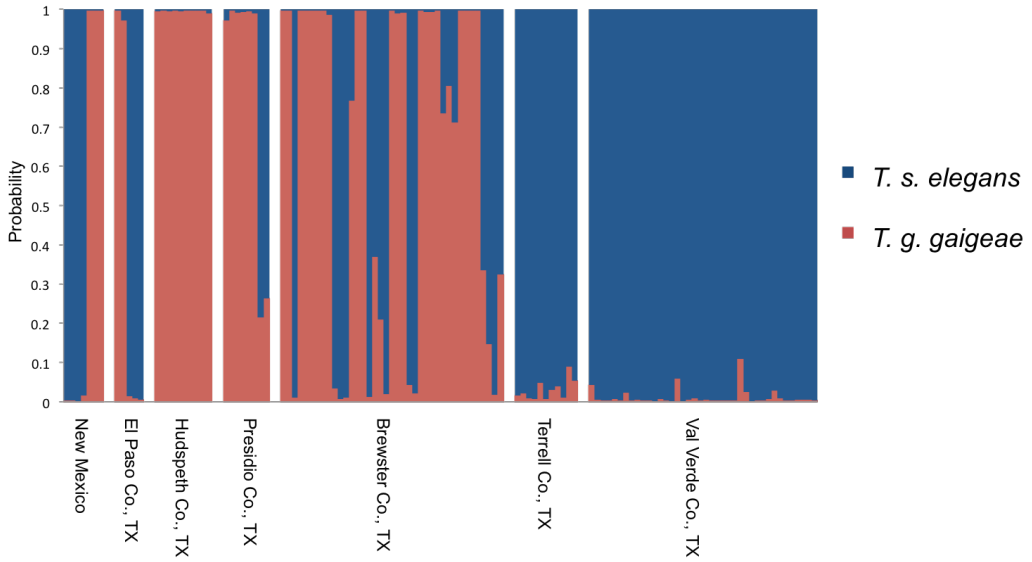

Figure 3. Assignment probabilities from *Structure* v2.3.4 when K=2 for each *Trachemys gaigeae gaigeae*, *Trachemys scripta elegans*, and suspected hybrid genotyped in this study. Each vertical line represents an individual. Individuals are organized based on geographic location with thick white lines separating each location. Individuals were assigned to one of the two species if they had 0.8 or greater assignment probability. All others were considered hybrids.

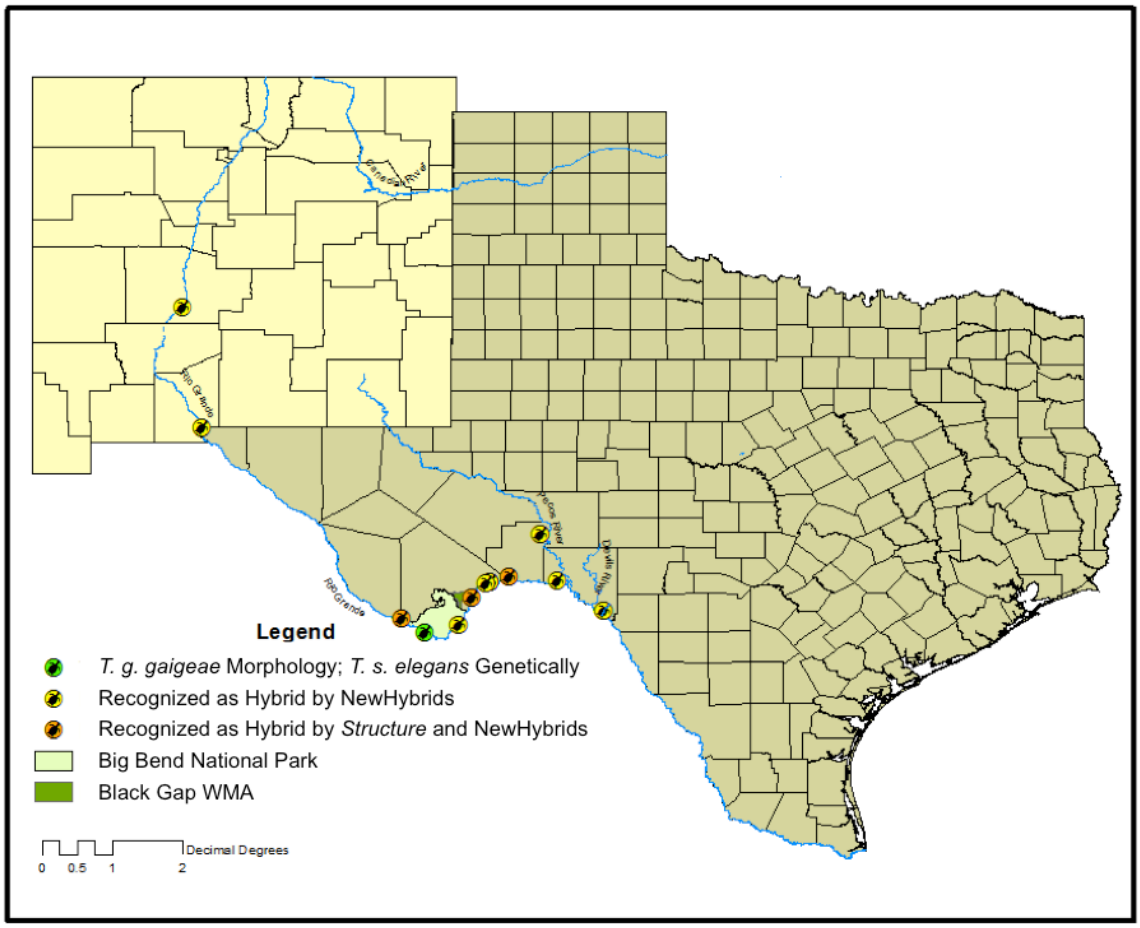

Figure 4. Locations of individuals recognized as hybrids of *Trachemys gaigeae gaigeae* and *Trachemys scripta elegans* in this study. Hybrids were found within or downstream of Big Bend National Park (3 in Big Bend National Park, 2 in Black Gap Wildlife Management Area, and 8 in the Lower Canyons) or near tributaries of the Rio Grande (5 in Oasis Ranch, 1 in Langtry, TX, and 5 in Del Rio, TX). Additional hybrids were located in New Mexico (1), El Paso, TX (2) and Lajitas, TX (2).

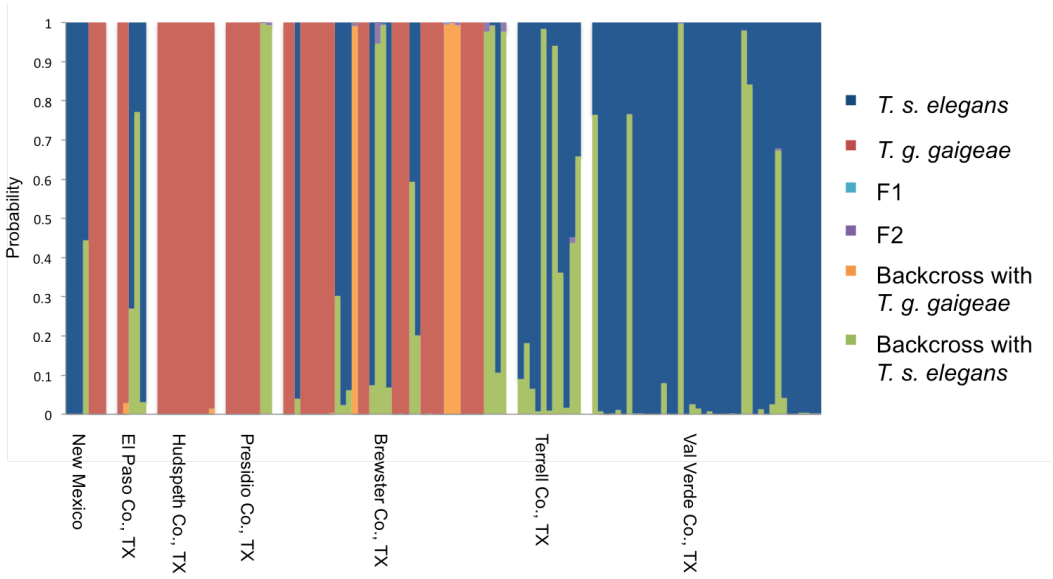

Figure 5. Average assignment probabilities from NewHybrids v1.1 for each *Trachemys gaigeae gaigeae*, *Trachemys scripta elegans*, and suspected hybrid genotyped in this study. Each vertical line represents an individual. Individuals are organized based on geographic location with thick white lines separating each location. Individuals were assigned to one of the two species if they had 0.8 or greater assignment probability. All others were considered hybrids.

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