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USING MOLECULAR MARKERS TO ASSESS SPECIES DISTRIBUTION, CONTACT ZONES, AND HYBRIDIZATION IN OKLAHOMA POCKET GOPHERS (*GEOMYS*)

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By

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USING MOLECULAR MARKERS TO ASSESS SPECIES DISTRIBUTION, CONTACT ZONES, AND HYBRIDIZATION IN OKLAHOMA POCKET GOPHERS (*GEOMYS*)

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ABSTRACT OF THESIS

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TITLE OF THESIS: Using Molecular Markers to Assess Species Distribution, Contact Zones, and Hybridization in Oklahoma Pocket Gophers (*Geomys*)

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ABSTRACT: Speciation is the evolutionary process which leads to the formation of new and distinct species. Understanding the mechanisms involved in the speciation process is imperative for understanding evolutionary biology and species diversity. A promising approach to understanding the mechanisms involved in speciation is the study of hybrid zones where genetic exchange between distinct species can produce mixed or recombinant genotypes. Analysis of hybrid zones can provide insight into the processes that isolate groups from each other.

Cryptic species are those that are difficult to identify based on morphological characteristics, but are reproductively or genetically distinct. A less restrictive, but similar term, is species complex. A species complex contains two or more closely related species with similar morphologies that have species boundaries that are difficult to define. Both of these terms can be applied to the genus *Geomys* (pocket gophers). Because of the morphological similarities between species in this genus, molecular analyses often are necessary for species identification. Currently, two species of *Geomys* are known to occur in Oklahoma. *Geomys bursarius* (plains pocket gopher) occurs in western Oklahoma,

whereas *G. breviceps* (Baird's pocket gopher) occurs in eastern Oklahoma. There has been limited molecular research done on Oklahoma *Geomys*, which has led to a lack of understanding of species boundaries throughout the state. Hybridization between *G. bursarius* and *G. breviceps* has been reported to occur in Oklahoma where the two species come into contact, which can further complicate species identification. Additionally, the identification of pocket gophers in the Oklahoma panhandle is in question. The panhandle populations are presumed to belong to *G. bursarius*, but it has been hypothesized that a third species, *G. jugossicularis* (Hall's pocket gopher), may reside there as well. The goals of my research were to utilize molecular markers to 1) determine if *G. jugossicularis* occurs in the Oklahoma panhandle, 2) evaluate the boundary line between *G. bursarius* and *G. breviceps* in central Oklahoma, and 3) determine the location of contact zones in the state and assess whether hybrid individuals occur within these zones.

The complete mitochondrial cytochrome-*b* gene was used to aid in species identification. Microsatellite markers were used to identify unique genetic clusters in Oklahoma, address the possibility of hybridization in Oklahoma, as well as evaluate genetic diversity within and between species.

Geomys jugossicularis was identified in the Oklahoma panhandle (Beaver and Cimarron Counties) as well as in the Texas panhandle in Dallam and Hartley Counties. Four admixed individuals between *G. jugossicularis* and *G. bursarius* were identified in Beaver and Cimarron Counties. The boundary line between these two species most likely meanders in and out of the Oklahoma and Texas panhandles, although additional research is needed to determine the extent and location of the contact zone between these two

species in this region. The boundary line between *G. bursarius* and *G. breviceps* was found in central Oklahoma and is similar to that proposed based on morphological data. It is likely that multiple contact zones exist along the boundary. Contact zones were evaluated in Tulsa and Cleveland Counties and it was determined that contact zones may be wider than originally suggested. Four admixed individuals were identified in Seminole, Logan, and Marshall Counties. Based on findings from this project and previous research, the tension zone model is likely the best fit model to describe the maintenance of hybrid zones in central Oklahoma.

The overall goals of this research were to gain a better understanding of species boundaries, including the locations of contact zones; hybridization; and genetic diversity within and among Oklahoma pocket gophers. My research allowed an opportunity to advance the growing knowledge of cryptic species and the occurrence of hybridization between species. Most importantly, my project has contributed to a broader understanding of *Geomys* in Oklahoma, which will allow for future research opportunities. Future research will focus on characterizing individuals using the whole genome approach as well as studying hybrid zones in more detail. With use of the whole genome approach, future research will be able to determine which regions of the genome are under selection in each hybrid zone. By determining common characteristics among zones, there will be a better understanding of the mechanisms necessary for speciation.

CHAPTER 1

USING MOLECULAR MARKERS TO EVALUATE CONTACT ZONES AND HYBRIDIZATION IN CRYPTIC SPECIES

The process of speciation

Reproductive isolation (the lack of gene flow between populations) is important in species because, in the absence of genetic exchange, different patterns of selection, genetic drift, and mutation will lead to evolutionary differences between groups (Pfennig and Pfennig 2012). Speciation, the formation of new species, relies on the establishment of reproductive and genetic barriers between two populations. By contrast, when two groups exchange alleles via interbreeding, evolutionary differences do not accumulate (Pfennig and Pfennig 2012).

To fully understand the complex mechanisms that drive speciation, it is necessary to gain knowledge of the reproductive and ecological properties of each group as they become genetically isolated (Slatkin 1987). The mechanisms that play a role in reducing gene flow can be studied in areas where these barriers have not yet been completed, such as hybrid zones (Alexandrino et al. 2005). Hybrid zones can represent several stages of population divergence in the process of speciation (Hewitt 1988). Because two genetically distinct groups are mating in a hybrid zone, there is a wide range of genotypes occurring throughout the zone, including recombinant genotypes (Barton and Hewitt 1985; Slatkin 1987). These genotypes can aid in the understanding of genetic divergence and speciation (Barton and Hewitt 1985). Therefore, hybrid zones can provide insight into the mechanisms that isolate groups from one another.

Contact zones and hybrid zones

Contact zones are regions where two genetically distinct species overlap and have the potential to produce offspring of mixed ancestry. (Barton and Hewitt 1985). Contact zones can be influenced by a variety of factors such as vegetation, climate, and geological variation (Gay et al. 2008). What role gene flow plays depends both on the geographic distribution of the species in question and the effects of other evolutionary forces such as selection (Slatkin 1987).

A contact zone can form in one of two ways. A primary contact zone is formed when previously sympatric (geographically overlapping) populations diverge. Over time, as the populations spread geographically, they come back into contact with one another and hybridization may follow. Secondary contact zones are those in which previously allopatric (non-overlapping) populations come into contact and have the potential to interbreed (Hewitt 1988). It can be difficult to determine how a contact zone formed because primary and secondary contact can produce the same patterns of variation (Harrison 1993). However, the majority of contact zones are thought to form through secondary contact, because most species are hypothesized to arise through allopatric speciation (Hewitt 1988; Harrison 1993; Arnold and Hodges 1995).

If reproduction between species occurs within the contact zone, it can be considered a hybrid zone. Once hybrid zones are formed they can be either stable or ephemeral (Harrison 1986). There are four models that commonly are used to explain the structure and maintenance of a hybrid zone (Table 1.1). The ephemeral-zone model or adaptive species complex portrays hybrid zones as short-lived, resulting in either

complete reproductive isolation via reinforcement or the merging of two species through introgressive hybridization (repeated backcrossing of a hybrid with a parent species; Sibley 1954; Wilson 1965; Remington 1968).

The hybrid-superiority model assumes higher fitness in hybrids than the parent species in certain environments (Lewontin and Birch 1966). Under this model, hybridization must take place in either a unique environment from both parent species or in an ecotone. Under such conditions, the hybrid offspring will out-compete parental types and hybrid zones will be maintained in these unique habitats or ecotones.

The dynamic-equilibrium model states that hybrid zones may be stable, but restricted, because the two species have diverged to the point where hybrid offspring are less fit than the parental species (Moore 1977). As a result, gene flow through the hybrid zone to parent populations would be repressed by selection. In areas with steep selection gradients, the hybrids would be confined to a narrow range of habitat between the two parental populations (Moore 1977). This means only a small portion of individuals near or in the hybrid zone would experience selective pressures against hybridization. A significant portion of the two populations would never experience this selection pressure because hybrids are restricted to the hybrid zone (Gay et al. 2008).

The tension zone model is used to explain regions of hybridization that are maintained by a balance between dispersal and selection against hybrid offspring (Gay et al. 2008). The key factor in the formation of a tension zone is that hybrid offspring have reduced fitness (Hewitt 1975). Gene flow between the two parental species is limited because of selective pressures against hybridized offspring (Nichols 1989). Tension

zones differ from dynamic-equilibrium zones because ecological factors do not influence them (Hewitt 1988; Nichols 1989; Gay et al. 2008). Therefore, tensions zones have been shown to be mobile in some populations due to the lack of environmental restrictions. Tension zones tend to form in areas of the contact zone with lower population densities (Nichols 1989). In species that have intermittent populations and only occasional gene flow between these populations, it is possible that two genetically distinct species remain separate and a narrow contact zone will remain indefinitely.

Hybridization and speciation

Hybridization is a phenomenon that occurs commonly among plants, fungi, and animals (Hewitt 1988; Seehausen 2004; Mallet 2007; Barton 2013). Plant hybridization has been well studied, because hybrid offspring easily replicate via asexual reproduction (Fritz et al. 1994; Whitney et al. 2010). Those species that do hybridize with one another are likely closely related (Seehausen 2004), though they are not always sister species. When interbreeding between two genetically distinct species takes place, it immediately creates variation in traits among hybrid offspring (Seehausen 2004).

Hybridization occurs in most proposed models of speciation (Abbott et al. 2013). When hybridization occurs between two species, the offspring typically are less fit than either parent species (Harrison 1986; Arnold and Hodges 1995; Barton 2013; Griebel et al. 2015). This is because genotypic combinations of the parental species have been shaped by generations of selective pressure, whereas hybrid offspring may be less adapted to the environment due to their mixed genotypes, and therefore hybrids can be outcompeted by the parental species (Griebel et al. 2015). However, it is possible for

hybrid offspring to out-compete parent species (Lewontin and Birch 1966; Harrison 1986; Seehausen 2004). When hybrids are more fit than parent species, increased phenotypic variation in hybrid populations can exceed the variation of both parent species (Lewontin and Birch 1966). This ultimately can lead to transgressive segregation which is caused by the formation of extreme phenotypes observed in the hybrid populations (Seehausen 2004). Understandably, speciation does not occur every time two species hybridize.

Cryptic species

Traditionally, the scientific community has accepted morphological differences and reproductive isolation between two groups as indicators of species identification. These characteristics have served as the basis for recognition among most mammalian species with allopatric geographic distributions (Baker and Bradley 2006). However, with the advent of modern genetics it has been discovered that some groups are genetically distinct but not morphologically unique (Baker and Bradley 2006). Cryptic species are two or more lineages that are not easily identifiable using morphological characteristics but are reproductively or genetically distinct (Bickford et al. 2007). This raises questions regarding how to characterize groups that are not morphologically divergent, particularly those that may hybridize.

Cryptic species evolve with little morphological divergence, which provides the opportunity to study the evolutionary processes that result in phenotypic conservatism (Smith et al. 2011). There are three mechanisms that can be used to explain evolution

without morphological changes: neutral genetic drift, constrained evolution, and correlated evolution (Smith et al. 2011).

Neutral theory was introduced by Kimura (1968, 1983) and is built on the assumption that on a molecular level, evolutionary changes within and between species are primarily caused by genetic drift and selectively neutral mutations and not by natural selection. Therefore, variation among closely related lineages is not formed through natural selection, but through neutral, nonadaptive processes (Bostwick and Brady 2002). Consequently, phenotypic and genotypic differences are expected to form proportionally throughout time in populations of cryptic species that are maintained by neutral drift (Lynch 1990; Smith et al. 2011).

Constraints in evolution can lead to phenotypic stasis through various processes. For example, populations can retain morphological features for long periods of time due to stabilizing selection toward an optimal phenotype (Charlesworth et al. 1982). Developmental constraints also can play a role in phenotypic conservatism among populations. Organisms can only produce so many variable phenotypes and the effects of natural selection will favor some phenotypes while selecting against others (Smith et al. 1985). When extreme phenotypes are selected against, stabilizing selection prevents divergence in morphology and function (Smith et al. 2011). This means that populations can remain morphologically stable for long periods of time.

Correlated (convergent) evolution explains the processes involved in two or more similar traits occurring across multiple species and may be involved in forming similar characteristics among cryptic species (Smith et al. 2011). Correlated evolution assumes

that the analogous traits evolved independently in multiple species (Pagel 1994). These traits most likely formed due to responses to specific habitats (Smith et al. 2011). As a result, cryptic lineages that evolved in comparable ecological niches may have developed similar traits that are adapted to their surroundings (Pfenninger et al. 2003). Comparable morphology among cryptic species undergoing correlated evolution is strongly associated with specific habitat type and not phylogeny (Harvey and Pagel 1991; Smith et al. 2011).

Regardless of how they arise, failure to detect cryptic species can have negative consequences, especially for conservation and management (Bickford et al. 2007). Baker and Bradley (2006) have shown that morphological classification of museum voucher specimens failed to identify a number of cryptic species that were later identified using genetic analyses. Thus, genetic studies are required to identify morphologically indistinguishable species. There are a variety of genetic markers that can be used to determine cryptic species identity, as well as compare genetic diversity and differentiation between species, and evaluate contact zones and hybridization.

Species identification using genetic markers

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are tandemly repeated DNA sequences found throughout the genome, particularly in eukaryotes where they are abundant (Viguera et al. 2001). Repetitive sequences, including microsatellites, typically are the most variable sequences in a genome (Ellegren 2004). The repeat units of microsatellites usually range between 1-6 base pairs (Chistiakov et al. 2006). Microsatellites are codominant, single locus markers that are small in size (Chistiakov et al. 2006), highly variable with regards to allelic

diversity (Ellegren 2004; Chistiakov et al. 2006), often have high heterozygosity (Ellegren 2004), and are easily amplifiable via polymerase chain reaction (PCR; Chistiakov et al. 2006). These features of microsatellites can be applied to an extensive range of questions in both basic and applied biology (Chistiakov et al. 2006).

Mutations during the replication process lead to variation in microsatellite markers which make them useful for individual identification. Length changes in microsatellite DNA usually arise from replication slippage (Ellegren 2004). Replication slippage is a type of mutation that leads to either expansion or contraction of the repeat unit during DNA replication (Ellegren 2004), resulting in variation in the number of repeat copies in daughter strands (Viguera et al. 2001). These variations create different allele lengths allowing for comparisons within and between populations. Additionally, microsatellites can be used to evaluate genetic diversity and differentiation between species.

Mitochondrial DNA (mtDNA) has been a common marker used to assess molecular diversity in animals for over 30 years (Galtier et al. 2009). Mitochondrial DNA is a useful marker for addressing molecular diversity due to a variety of reasons (Galtier et al. 2009). The mitochondrial genome is maternally (clonally) inherited, meaning the genome does not undergo recombination and is therefore passed intact to all offspring. Offspring share a common linkage which allows for a simplified representation between species. This common linkage between species allows for easier analysis of variation within and between populations. The evolutionary rate of change of mtDNA has been presumed to be clock-like. In the absence of any mutations spreading through positive selection, only neutral mutations accumulate in time and divergence levels should reflect

divergence time; therefore, the mitochondrial genome is ideal for phylogenetic analyses. Additionally, the lack of genetic exchange and common genealogy also makes mtDNA a useful species identification marker (Avise et al. 1987). There are a large number of copies of mtDNA present in each cell which significantly raises the sensitivity of analyses (Branicki et al. 2003). These characteristics of mtDNA make it an ideal marker for understanding population and species history

One of the regions of mtDNA commonly used for species identification and phylogenetic studies, particularly in mammals, is the cytochrome-*b* (*Cytb*) gene (Hsieh et al. 2001; Branicki et al. 2003). The *Cytb* gene is useful for species identification because a large database of sequences is available for comparison (e.g., GenBank). This gene was used to determine the initial genetic identification of specimens collected in this study using restriction fragment length polymorphism (RFLP) and phylogenetic analyses.

Restriction fragment length polymorphism is a technique that utilizes restriction enzymes for various analyses such as DNA fingerprinting, paternity testing, and genetic diversity (Butler 2009). Restriction enzymes, also known as restriction endonucleases, are enzymes that cut DNA at specific regions (Wolf et al. 1999). There is variation in restriction sites between species that allows for species identification. In this study, PCR-RFLP was utilized to analyze the mitochondrial *Cytb* gene and determine species identification.

Phylogenetic analyses are useful in understanding evolutionary relationships among closely related species (Holder and Lewis 2003). Phylogenetic analysis also can aid in species identification through the construction of phylogenetic trees and assignment of unknown samples to clades (e.g., DNA barcoding). The estimation of

pairwise distances, which is the comparison of genetic differences between individuals, also can aid in the understanding genetic distances among taxa (e.g., Bradley and Baker 2001; Sudman et al. 2006). Phylogenetic trees are constructed by comparing sequences of individuals within a taxon or across taxa using specific models of evolution.

Pocket gophers

Pocket gophers (Geomyidae) are medium-sized, fossorial rodents whose diets primarily consist of grasses (Foster and Stubbendieck 1980). Pocket gophers get their name from their fur-lined cheek pouches which they use for vegetation storage while foraging. Pocket gophers range in size from 150 to 250 mm, with a short hairy tail (25-50 mm). Most gophers have brown fur which closely resembles the soil in the habitat they are occupying (Krupa and Geluso 2000). There are three genera of pocket gophers that are found in a wide range across the United States as well as northern Mexico. *Thomomys* (smooth-toothed pocket gophers) occur in the southwestern portion of the United States from California to Colorado, with range extensions into northern Mexico (Reid 2006). *Cratogeomys* (yellow-faced pocket gophers) have a much smaller range than *Thomomys* and occur primarily in the south-central United States as well as northern Mexico (Reid 2006). Members of the genus *Geomys* (plains pocket gophers), the focus of this research, primarily occur in the central plains of the United States, with small range extensions into south-central Canada, the eastern coast of Mexico, and the southeastern United States (Hall 1981).

Pocket gophers occur in a limited range of soil and vegetation types throughout their distribution (Foster and Stubbendieck 1980). Soil type heavily influences the distribution of pocket gophers, with many species preferring sandier soils (e.g.,

Downhower and Hall 1966). Pocket gophers tend to avoid clay soils and soils that contain a high percentage of gravel or stone (Davis and Schmidly 1994). Low mobility, fossorial habits, and dependence on specific soil types have resulted in geographical patterns that reflect the isolated nature of pocket gopher populations (Mauk et al. 1999). These patterns of isolation have played a role in the taxonomic history of pocket gophers.

Geological history also has had an impact on the species distribution of pocket gophers in the grasslands of the Great Plains. Serial glaciation, particularly in the Wisconsin Era (Blair 1954), has been proposed as a primary factor driving divergence and speciation of gophers in the Great Plains. The advance and retreat of glaciers produced isolated populations, resulting in numerous independent speciation events in this region (Mauk et al. 1999).

The geologic history of the region, as well as the natural history of pocket gophers, makes *Geomys* populations in the Great Plains an ideal system for understanding divergence and speciation mechanisms, particularly those related to isolation and secondary contact after isolation. For example, the diverse soil types in Texas are associated with small isolated populations of pocket gophers throughout the state (Mauk et al. 1999). Pocket gophers have been heavily studied across Texas and there is a great understanding of species boundaries and contact zones. Few molecular studies of pocket gophers have occurred in Oklahoma, and as a result there is poor resolution of species boundaries and species diversity in the state.

The pocket gophers found in the Oklahoma panhandle are presumed to belong to *G. bursarius major*; however, Genoways et al. (2008) suggested the possibility that a

newly elevated species of pocket gopher, *G. jugossicularis* (Hall's pocket gophers), may extend into the Oklahoma and Texas panhandles. Therefore, species boundaries and contact zones of the Oklahoma and Texas panhandles need to be studied to have a better understanding of species distribution in this region.

The most extensive study of Oklahoma pocket gophers was conducted by Heaney and Timm (1983) who used cranial measurements to determine the boundary line between *G. bursarius* (plains pocket gophers) and *G. breviceps* (Baird's pocket gophers) runs north and south in central Oklahoma. Heaney and Timm (1983) also proposed that along the boundary line there may be multiple contact zones and possible hybridization between the two species. However, because morphological characteristics were used to determine the distribution and boundary line between these two cryptic species, molecular analyses need to be conducted to confirm these results, as well as evaluate potential hybridization between these species.

The overall objectives of my research were to assess genetic diversity and species boundaries as well as to evaluate contact zones and hybridization in Oklahoma pocket gophers (*Geomys*). My project focused on two regions. The first part of this project (Chapter 2) examined the Oklahoma and Texas panhandles to address the possibility of a cryptic species of pocket gopher, *G. jugossicularis,* occurring in the Oklahoma panhandle. The goals of this chapter were to 1) determine if *G. jugossicularis* occurs in the Oklahoma and Texas panhandles and 2) determine the range of the species in the panhandle regions. The second part of my project (Chapter 3) was concentrated in central Oklahoma. The goals of this portion of the research were to 1) determine the boundary line between *G. bursarius* and *G. breviceps* in central Oklahoma, 2) identify contact

zones between the two species, and 3) identify possible hybrid zones. Chapter 4 focuses on the overall findings from this project and discusses future research that can be conducted now that a wide range of Oklahoma pocket gophers have been genetically evaluated.

Table 1.1: Comparison of hybrid maintenance models.

	Reduced Hybrid Fitness	Stable	Influenced by Ecological Factors
Ephemeral Zone	Yes	No	No
Hybrid- Superiority	No	Yes	Yes
Dynamic-Equilibrium	Yes	Yes	Yes
Tension Zone	Yes	Yes	No

CHAPTER 2

EVALUATION OF POCKET GOPHERS IN THE OKLAHOMA AND TEXAS PANHANDLES

INTRODUCTION

Species are the fundamental unit for understanding ecology, biodiversity, and conservation efforts (Bradley and Baker 2001). Traditionally, species have been identified based on morphological features (see Bradley and Baker 2001; Bickford et al. 2006; Smith et al. 2011). With the increasing use of molecular markers to evaluate linages, researchers have determined that identifying species based on morphological characteristics can result in the misidentification of cryptic species (e.g., Baker and Bradley 2006).

Cryptic species are those that are difficult, and in some cases impossible, to distinguish from one another based on traditional morphological traits (Baker 1984; Baker and Bradley 2006; Pfenninger and Schwenk 2007). Species from a wide range of taxa have been found to display cryptic characteristics (Bickford et al. 2006). Accurate species identification is crucial for conservation efforts, diagnosis and prevention of disease, and the identification of invasive and pest species (Bickford et al 2006). Additionally, identification of cryptic species is critical for basic research where the presence of more than one species in a sample set may impact the interpretation of hypotheses and the direction of future research (Baker 1984). In order to understand biodiversity, all species must be identified and their distributions and boundaries should be documented.

Pocket gophers of the genus *Geomys* are cryptic species that are difficult to identify using morphological characteristics. *Geomys* are found throughout the central plains of the United States, with small range extensions into southern Canada, northern Mexico, and the southeastern United States (Hall 1981). While well studied in certain parts of their range, there is limited research involving Oklahoma pocket gophers. The most extensive study of Oklahoma pocket gophers was conducted by Heaney and Timm (1983). Cranial measurements were used to conclude there are two species of *Geomys* in Oklahoma: *G. bursarius* (plains pocket gopher) in western Oklahoma and the panhandle, and *G. breviceps* (Baird's pocket gopher) in eastern Oklahoma.

The identification of gophers in the Oklahoma panhandle has been somewhat contentious, with little research performed in this region. Using preserved skins and cranial measurements, Hooper (1940) concluded a subspecies of *G. bursarius* (then referred to as *G. lutescens*), *G. l. jugossicularis*, occurred in the Oklahoma panhandle. However, panhandle samples currently are considered to belong to *G. bursarius major*, the same subspecies found throughout western Oklahoma (Hall 1981). As part of a larger evaluation of the systematics of *Geomys*, Sudman et al. (2006) suggested recognizing *G. bursarius jugossicularis* as a distinct species, *G. jugossicularis* (Hall's pocket gopher). Genoways et al. (2008) recognized the validity of *G. jugossicularis* as a distinct species in a study focused on gopher populations in Nebraska, and suggested its distribution may extend as far south as the Oklahoma (Cimarron Co.) and the Texas panhandles, based on historical taxonomic distributions associated with the name. As part of another large-scale evaluation of pocket gopher taxonomy, Chambers et al. (2009) confirmed the validity of *G. jugossicularis* as a distinct species, but did not suggest its range reached the

Oklahoma and Texas panhandles. To date, there have been no genetic studies evaluating the possibility of *G. jugossicularis* in the Oklahoma and Texas panhandles.

The goals of my research were to use molecular markers to 1) determine if the range of *G. jugossicularis* includes the Oklahoma and Texas panhandles as suggested by Genoways et al. (2008), 2) determine the extent of *G. jugossicularis'* range in Oklahoma if it is present, and 3) assess genetic diversity within species and genetic differentiation between species found in western Oklahoma and the panhandle regions.

METHODOLOGY

Sample collection

I obtained 54 specimens from 11 counties in and around the Oklahoma and Texas panhandles (Fig. 2.1). I also obtained samples from within the well-defined range of *G. bursarius* in western Oklahoma for comparison to the panhandle samples. When available, I obtained tissues from museum collections (Appendix 2.1). Specimens were collected using Victor and Macabee gopher kill traps, and a combination of chloroform and thoracic compressions were used to euthanize the animals if necessary. This protocol is approved by the American Society of Mammalogists (ASM) IACUC (Sikes et al. 2016) as well as The University of Central Oklahoma (UCO) IACUC (#14011).

I collected samples from both public and private properties. All trapping completed on private property was conducted with landowner's permission. Upon capture, each specimen was assigned an identification number. I recorded geographic

coordinates of each sample in the form of latitude and longitude using a hand held GPS (Appendix 2.1). Specimens were immediately stored on ice before being transported to the lab for processing. During processing, I recorded standard measurements and sex for each specimen and kidney, heart, liver, lung, muscle, spleen, and colon tissues were collected from each sample. All specimens and tissue samples were deposited in the UCO Natural History Museum.

DNA extraction, PCR amplification, and genotyping

I extracted DNA from liver samples using DNeasy Blood and Tissue Extraction kits (Qiagen). I measured nucleic acid concentrations using the NanoDrop 2000 (ThermoFisher Scientific) to ensure high quality, intact DNA was being used for all genetic analyses. I used the S100 Thermal Cycler (Bio-Rad) to perform polymerase chain reaction (PCR) and cycle sequencing. I visualized PCR or cycle-sequenced products using an ABI3130 or ABI3500 Genetic Analyzer (ThermoFisher Scientific). I visualized, edited, and scored microsatellite genotypes using GeneMapper Software 5 (ThermoFisher Scientific). I aligned and edited sequences with Sequencher 4.10.1 (Gene Codes Corporation).

I amplified the complete mitochondrial DNA (mtDNA) cytochrome-*b* (*Cytb*) gene for all samples using two amplification primers (Table 2.1). The total PCR reaction volume was 25 µL, which consisted of 12.5 µL nuclease free water, 8.3 µL failsafe premix C (Epicentre), 1.0 µL of each amplification primer, 0.2 µL GoTaq Flexi DNA polymerase (Promega), and 2 µL of DNA. PCR thermal cycling parameters were initial denaturation at 94°C for 2 minutes; followed by 38 cycles of 92°C for 15 seconds, 54°C

for 1 minute, 72°C for 1 minute and 10 seconds; and a final 72°C extension for 10 minutes.

I sequenced the *Cytb* gene for 11 samples collected in the Oklahoma panhandle using six sequencing primers (Table 2.1). Samples were cycle sequenced using the Big Dye Terminator version 1.1 (B. D. v1.1) Cycle Sequencing Kit (ThermoFisher Scientific). The cycle sequence reactions had a total volume of 10 µL which consisted of 4.7 µL nuclease free water, 1.5 µL BigDye 5X sequencing buffer, 1.0 µL B. D. v1.1 Ready Reaction Mix, 0.8 µL sequencing primer, and 2.0 µL PCR product. PCR thermal cycling parameters included 25 cycles of 96°C denaturation for 10 seconds, 50°C annealing for 10 seconds, and 60°C extension for 2 minutes. I used the Performa DTR gel filtration kit (EdgeBio) for purification of the cycle sequencing products. Samples were prepared for the genetic analyzer with a reaction mixture that contained 10 µL Hi-Di formamide (ThermoFisher Scientific) and the total purified cycle sequenced product volume (~10 µL).

I used nine microsatellite primer pairs (Table 2.2) to genotype all samples of *Geomys* collected for this project. Welborn et al. (2011) developed six of the nine primers specifically for *G. breviceps*, and these primers have been shown to successfully amplify DNA in other *Geomys*. The remaining three primers were developed for *Thomomys* (smooth-toothed pocket gophers; Steinberg 1999) and have been shown to amplify DNA in *Geomys* (Welborn et al. 2011). PCR amplifications were performed in 25 µL reaction volumes containing 14.3 µL nuclease free water, 5 µL 5X colorless GoTaq buffer (Promega), 2 µL $MgCl_2$ (Promega), 0.75 µL 10 mm mixture of dNTPS (New England Biolabs), 0.6 µL of each primer, 0.25 GoTaq Flexi DNA polymerase, and 1.5 µL DNA.

Annealing temperatures differed across loci and can be found in Table 2.2. The general PCR thermal cycling parameters were as follows: initial denaturation at 95°C for 3 minutes; 9 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, and 72°C extension for 1 minute; 9 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, and 72°C extension for 1 minute; 14 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, 72°C extension for 1 minute; and a final 72°C extension for 10 minutes. The samples were prepared for the genetic analyzer using 9.25 µL Hi-Di formamide, 0.25 µL size standard (Genescan ROX 500; ThermoFisher Scientific), and 0.5 µL PCR product.

Cytochrome-b data analysis

To determine species identification, I constructed phylogenetic trees to compare 11 Oklahoma panhandle samples to 32 *Geomys* samples obtained from GenBank (Appendix 2.2). One *Cratogeomys castanops* sample was used as the outgroup. I used Molecular Evolutionary Genetic Analysis (MEGA) 7 (Kumar et al. 2016) to align the complete sequences via MUSCLE, determine the most appropriate model of evolution for phylogenetic analyses, perform maximum likelihood (ML) analyses, and estimate pairwise distances. Due to low numbers of informative characters, all codon positions were analyzed together. I used 1000 bootstrap pseudoreplicates for the ML analysis. The model test indicated that the Hasegawa-Kishino-Yano model with proportion of invariant sites, and gamma distribution parameters (HKY+I+G) best fit the dataset. The HKY model assumes nucleotides occur at different frequencies. This model also assumes that nucleotide substitutions such as transitions and transversions occur at different rates

(Hasegawa et al. 1985). The proportion of invariant sites model estimates some proportion of sites that do not change across the in-group over the course of evolution (Steel et al. 2000). The gamma parameters allow the analysis to estimate different evolutionary rates for different sites (Yang 1994). Pairwise distances between individuals were calculated in MEGA using the suggested Tamura-Nei (TN3) model with gamma distribution. The TN3 model assumes the transversions occur at the same rate, but transitions can occur at different rates (Tamura and Nei 1993). I performed Bayesian Inference (BI) analyses using BEAST 1.8.2 (Drummond et al. 2012). The Yule process and a relaxed uncorrelated lognormal clock were used. I performed these analyses using a 10% burn-in with 100 million generations. There were 20 million generations with sampling every 10,000 trees.

I also used restriction fragment length polymorphism (RFLP) to determine species identification for all samples collected for this project. This technique utilizes restriction enzymes to fragment DNA and produce unique banding patterns. Restriction enzymes, also known as restriction endonucleases, are enzymes that cut DNA at specific sequences (Wolf et al. 1999). Once variation in restriction sites between species is found, this technique can be used for species identification (Avise et al. 1979; Baker et al. 1989). BamHI (New England Biolabs) is a restriction enzyme that was isolated from *Bacillus amlyiqufaciens* and can be used to distinguish between *G. bursarius* and *G. jugossicularis. G. bursarius* has no cut site, resulting in a fragment of 1140 base pairs (bp), the total length of the *Cytb* gene. *G. jugossicularis* has one cut site which results in two restriction fragments of 530 bp and 610 bp in length.

Once samples were amplified for the *Cytb* gene, BamHI was added to each sample for RFLP analysis. The manufacturer's protocol was followed and included 3 µL of PCR product, 5 µL of 10X NEbuffer (New England Biolabs), 1 µL enzyme, and 41 µL of nuclease free water for a total reaction volume of 50 µL. Samples were incubated for 1 hour at 37°C. Gel electrophoresis was performed on a 2% agarose gel using 0.5X TBE (Tris/Borate/EDTA) buffer. The electrophoresis rigs were set to 120 volts and run until the samples migrated halfway down the gel. Gels were viewed using the UVP GelDoc-It 310 Imaging System (ThermoFisher Scientific). Each sample was visually assigned a species identification based on the number and length of fragments.

Microsatellite data analyses

To complement the mtDNA data and provide further support for species identification, I used nuclear DNA to determine the number of genetically distinct groups occurring in the panhandles and western Oklahoma. Structure 2.3.4 (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009) is a program that uses allelic frequencies to estimate population structure. This program infers the presence of genetically distinct clusters occurring in the represented data by minimizing Hardy-Weinberd equilibrium (HWE) within clusters and assigns individuals to these genetically distinct clusters based on allelic frequencies. One Structure run was performed using microsatellite data for all samples (n = 54) and the following parameters: allele frequency correlated option, 100,000 burn-ins, 1,000,000 MCM chains, K = 1-5 (the number of possible clusters tested), and five iterations. The results obtained from Structure were then uploaded to Structure Harvester (Earl and vonHoldt 2012) to determine the most likely value of K that best fit the data based on the Evanno et al. (2005) method.

I used the Cluster Matching and Permutation Program (CLUMPP) v1.1.2 (Jakobsson and Rosenberg 2007) to average the five independent runs for each value of K. The FullSearch algorithm was used to determine the most likely probability that a given individual was assigned to a specific cluster by averaging the five result groups. Once the most likely cluster assignment for each individual was determined, the results were coalesced into a single output file. The output file produced by CLUMPP was used to construct a barplot for data visualization using the distruct v1.1 program (Rosenberg 2004).

I used ArcGIS (ESRI 2011) to create maps of the results. The dataset included all individuals and their location (longitude and latitude) as well as the cluster to which they were assigned. A single point feature class was created for the geodatabase. The NAD 1983 coordinates system and the United States Census Bureau cartographic boundary shapefile were used. A map of the PCR-RFLP results also was created using the same methods.

To make sure there were no genotypic scoring errors before additional analyses were conducted, I used MICROCHECKER v2.2.1 (Van Oosterhout et al. 2004) to determine if there were null alleles, large-allele dropout, or stutter-induced typing errors at each locus. This program randomizes the observed alleles for each locus in each individual sample to construct random genotypes. These random genotypes are then compared to the observed genotypes to determine the frequency of allele-specific homozygote size classes and allele-specific heterozygote size classes. The cumulative binomial distribution (Weir 1996) was used to determine the probability of both classes. The observed classes are then compared to the probability values to determine if there are

any genotyping errors. Because this dataset potentially included multiple species, the data was analyzed three ways: 1) all samples collected (n = 54), 2) samples identified as *G. bursarius* based on PCR-RFLP or cluster analyses (n = 35), and 3) samples identified as *G. jugossicularis* based on PCR-RFLP or cluster analyses (n = 19). These runs were conducted including the missing data values.

I used FSTAT 2.9.3.2 (Goudet 1995, 2001) and CERVUS 3.0.7 (Marshall et al. 1998; Slate et al. 2000; Kalinowski et al. 2007, 2010) to determine genetic diversity within and genetic differentiation between species. I used FSTAT 2.9.3.2 to assess the data for deviations from HWE and linkage disequilibrium as well as to estimate allelic diversity and F_{st}. Prior to this analysis, I organized samples according to RFLP species identification and Structure results, and admixture individuals were removed for clearer interpretation of the results. Three datasets were created for FSTAT analyses. The first two analyses divided samples identified as *G. bursarius* (n = 33, excluding admixture individuals) and samples identified as *G. jugossicularis* (n = 17, excluding admixture individuals). The third analysis combined all individuals (n = 50, excluding admixture individuals). In the combined dataset, samples were assigned to different "populations" based on species identification. Hardy-Weinberg was tested over all samples and population differentiation did not assume HWE. Hardy-Weinberg tests were performed per locus and sample. A pairwise test of differentiation also was performed in FSTAT. Genotypic equilibrium was tested between all pairs of loci and a 5/100 nominal level for multiple tests was used. The program CERVUS 3.0.7 was used to estimate observed and expected heterozygosity, polymorphic information content (PIC), and to perform allelic

frequency analyses. Samples for CERVUS analyses were divided into three datasets as previously discussed

RESULTS

RFLP analysis

Ninety percent of samples (49 of 54) were successfully identified using PCR-RFLP analyses (see Fig. 2.2 for representative gel). Nineteen samples were identified as *G. jugossicularis* and 30 samples were identified as *G. bursarius* (Appendix 2.1). I could not identify five samples using RFLP analysis. These samples included TK26732 (Moore Co., TX); OU3252 (Beaver Co., OK); and MSB273626, 273628, and 273629 (Caddo Co., OK; Appendix 2.1). A map depicting species distributions based on PCR-RFLP results is shown in Figure 2.3.

Sequencing analyses

The Bayesian inference tree recovered seven well supported "species group" clades [posterior probability (Pp) = 1, bootstrap values (Bs) ≥ 98; Fig. 2.4]. The first clade contained two subspecies belonging to *G. pinetus*. Clade 2 contained three samples of *G. breviceps*. Clade 3 contained *G. tropicalis* and *G. personatus*. This clade was sister to a clade that contained *G. attwateri* and *G. streckeri*, although the relationship between *G. attwateri* and G. *streckeri* was not supported. Clade 4 contained *G. knoxjonesi* and *G. arenarius*. Clade 5 consisted of three subspecies of *G. texensis*. Clade 6 consisted of individuals belonging to *G. bursarius*, including *G. bursarius major*, which is the

subspecies found throughout western Oklahoma. Clade 7 included samples that are within the proposed geographic range of *G. jugossicularis*. This clade included *G. bursarius halli*, *G. b. lutescens*, *G. jugossicularis halli*, *G. j. jugossicularis*, and the 11 samples from the Oklahoma panhandle. The *G. bursarius* samples obtained from GenBank that cluster in clade 7 likely represent misidentified samples. The 11 samples from the Oklahoma panhandle clustered together in a subclade that had strong bootstrap support (76) and a high posterior probability (1). This subclade was sister to *G. j. jugossicularis*, although there was no support for this relationship. The maximum likelihood tree showed a similar topology (data available from authors).

Pairwise distance estimations were used to determine the percent genetic difference between compared individuals. The average pairwise distance among all 11 samples collected from the Oklahoma panhandle was 0.2% (Table 2.3). When comparing the *G. j. jugossicularis* sample to the Oklahoma panhandle samples, the average difference was 1.9% difference. There was a 5.9% difference between the *G. b. major* individual obtained from GenBank and the panhandle samples (Table 2.3).

Genotyping errors

The MICROCHECKER analyses containing all individuals indicated that there was low heterozygosity across all loci, which could be a sign of null alleles. There was no evidence of allele dropout, but possible stuttering may have resulted in scoring errors for loci Gbr10, Gbr15, and Gbr27. The data set containing individuals identified as *G. bursarius* showed low heterozygosity across all loci. There was no evidence of allele dropout, but there could be possible error due to stuttering at loci Gbr10, Gbr15, and Gbr27. The dataset containing only individuals identified as *G. jugossicularis* showed

low heterozygosity in seven of the nine loci including Gbr09, Gbr10, Gbr25, Gbr33, Tm1, Tm6, and Tm7. There was no allele dropout, but possible error could have occurred due to stuttering at locus Tm1. All samples were scored twice across all loci before further analyses to ensure no user error occurred during genotype scoring or due to stuttering. Despite the low heterozygosity detected by MICROCHECKER, all loci were in HWE in all datasets (adjusted p-value = 0.00278).The adjusted p-value for genotypic disequilibrium was 0.001389. There were no pairs of loci found to be in linkage disequilibrium in any dataset.

Genetic structure

Results from Structure Harvester indicated the most likely scenario when all Oklahoma and Texas panhandle samples were included was K = 2 (Fig 2.5). This means there were two genetically distinct clusters occurring in the sampled range in the Texas and Oklahoma panhandles as well as western Oklahoma. The F_{st} value between the two clusters, estimated in FSTAT, was 0.160. An 80% threshold was used to determine admixed individuals, which means an individual sharing at least 21% of their alleles with one cluster and the remaining 79% with another cluster was considered an admixture individual. Three admixture individuals were identified in the Oklahoma panhandle. Two admixture individuals were found in Beaver Co., and one admixture individual was found in Cimarron Co. Cluster one consisted of 18 individuals found mostly in the Oklahoma panhandle and western portions of the Texas panhandle. Cluster two consisted of 33 individuals found in the far eastern Oklahoma panhandle, eastern Texas panhandle, and western Oklahoma. A map depicting the clustering results based on geographic locality is provided in Figure 2.6.

Genetic diversity

For the combined dataset, observed heterozygosity (HObs) was lower across all loci when compared to the expected value (mean HObs = 0.521; Table 2.4). The mean number of alleles per locus was 14.0, the mean expected heterozygosity was 0.851, and the mean PIC value was 0.823. Using the FSTAT output file, unique alleles were identified within the two species found in the Oklahoma and Texas panhandles. At locus Gbr09, six unique alleles were identified for *G. bursarius* and one unique allele for *G. jugossicularis*. At locus Gbr10, seven unique alleles were identified for *G. bursarius* only. For locus Gbr15, 11 unique alleles were identified for *G. bursarius* only. Six unique alleles for *G. bursarius* and one for *G. jugossicularis* were identified at locus Gbr25. At locus Gbr27, two unique alleles were identified for *G. bursarius* only. At locus Gbr33, *G. bursarius* had seven unique alleles and *G. jugossicularis* had one unique allele. At locus Tm1, *G. bursarius* had nine unique alleles and *G. jugossicularis* had one unique allele. For loci Tm6 and Tm7, eight unique alleles were identified for *G. bursarius* and one unique allele for *G. jugossicularis* was identified at both loci. Genetic diversity values for *G. jugossicularis* and *G. bursarius* are provided in Table 2.4.

DISCUSSION

Species identification

Using PCR-RFLP analysis, I was able to identify samples to species based on two distinct banding patterns (Fig. 2.2). Of the 54 samples collected in the panhandles and western Oklahoma, 49 individuals were identified to species using this technique.

Nineteen individuals had banding patterns expected of *G. jugossicularis* and 30 had banding patterns of *G. bursarius*. Using microsatellite markers and Structure, I was able to assign individuals to two genetically distinct clusters that did not have significantly overlapping distributions (Figs. 2.5 and 2.6). The five samples that could not be identified using RFLP analyses clustered with *G. bursarius* samples based on microsatellite data. When comparing the Structure results to the RFLP results (Appendix 2.1), the results mirrored each other with the exception of sample 3260. This individual was identified as *G. bursarius* based on RFLP analysis, but the Structure analysis clustered this individual with *G. jugossicularis*. This individual represents a putative hybrid (discussed below). Microsatellite data also was used to determine the F_{st} value between *G. bursarius* and *G. jugossicularis* (0.160), which indicated that there was a high level of genetic differentiation between the groups (Wright 1978).

Cytochrome-*b* sequence data also supported the identification of Oklahoma panhandle samples as *G. jugossicularis*. The topology of the cladogram (Fig. 2.4) was similar to the findings of Sudman et al. (2006). *Geomys bursarius major*, the subspecies found throughout western Oklahoma, clustered with clade 6, which includes other subspecies of *G. bursarius*. This clade was well supported (Pp = 1, Bs =99). Samples collected from the Oklahoma panhandle clustered in a separate clade (clade 7) that also was well supported (Pp = 1, Bs = 100). In addition to the Oklahoma panhandle samples, this clade contained *G. b. halli, G. b. lutescens, G. b. jugossicularis, and G. j. jugossicularis.* Individuals identified as subspecies of *G. bursarius* that clustered with *G. jugossicularis* in clade 7 were collected from within the proposed ranged of *G. jugossicularis* (Genoways et al. 2008). Additionally, the pairwise distance value between

G. jugossicularis samples and the *G. bursarius* subspecies clustered in clade 7 was 2.3% (Table 2.3). The most likely explanation for these results is misidentification of the samples due to the newly elevated status of *G. jugossicularis*. The clade containing *G. jugossicularis* is sister to a clade containing *G. bursarius* and *G. lutescens*, and this relationship is well supported (BS = 77, Pp = 0.99).

Sequence variation in the *Cytb* gene is a useful tool to determine what qualifies a group of individuals as a genetically distinct species when compared to other species (Bradley and Baker 2001). This value can be affected by several factors such as the taxa involved as well as the time since divergence between sister species of small mammals. Bradley and Baker (2001) found that distance values between subspecific taxa ranged between 0.009-2.34%. In this study, divergence values between sister taxa ranged from 4-11%. The average genetic distance between the samples collected within the Oklahoma panhandle was 0.2%, meaning these individuals most likely belonged to the same population/ or subspecies. When comparing the *G. j. jugossicularis* sample from GenBank to those collected in the panhandle, the average genetic distance value was 1.9%, which suggests the Oklahoma panhandle samples most likely belong to *G. jugossicularis*. When comparing the *G .b. major* sample from GenBank to the panhandle samples, the average distance value was 5.9%, which suggests they are genetically distinct from *G .b. major*. Based on previous studies (Bradley and Baker 2001), these values further support that the panhandle samples are genetically distinct from *G. b. major* samples. Taken together, all molecular data from this project supports the hypothesis that *G. jugossicularis* is a distinct species (Sudman et al. 2006; Genoways et

al. 2008; Chambers et al. 2009) and the presence of *G. jugossicularis* in the Oklahoma and Texas panhandles.

Range of G. jugossicularis in the Oklahoma and Texas panhandles

When examining the map of the RFLP and Structure results (Figs. 2.3 and 2.6, respectively), the individuals belonging to *G. jugossicularis* were restricted to the Oklahoma and Texas panhandles. Individuals identified as *G. jugossicularis* were found in Cimarron and Beaver Counties in Oklahoma, and Dallam and Hartley Counties in Texas. The individuals belonging to *G. bursarius* primarily were restricted to the western half of Oklahoma and the far eastern portion of the Oklahoma (Beaver Co.) panhandle, and large portions of the Texas (Moore Co. and Hemphill Co.) panhandle. Although samples were not collected from Texas County in the Oklahoma panhandle, it is likely that *G. jugossicularis* occurs in this county as well. Based on collection localities, it is possible that *G. jugossicularis* occurs in the adjacent Texas counties just south of the Oklahoma panhandle. Additionally, because admixture individuals were found in Beaver Co. and Cimarron Co., it is possible that the boundary line between the species runs through these counties. This means there could be multiple contact zones along the boundary line which is a possible explanation for the admixture individuals found in the Oklahoma panhandle. It also is possible that *G. bursarius* is expanding west or *G. jugossicularis* is expanding southeast into the panhandle, potentially along rivers. This might account for the distribution of admixture individuals seen in this region.

Hybridization

Though unexpected, it was not surprising that admixture individuals were found in the Oklahoma panhandle. Four admixture individuals were identified between *G.*

jugossicularis and *G. bursarius* (Figs. 2.5 and 2.6). Three admixtures (Beaver County: TK181066 and OU3259; Cimarron County, TK185661) were identified using Structure analyses (Appendix 2.1). Additionally, a putative hybrid individual was identified based on conflicting results from microsatellite and mtDNA analyses. Sample OU3260 (Beaver County) was identified as *G. jugossicularis* based on Structure analyses, but as *G. bursarius* based on PCR-RFLP analysis (Appendix 2.1). When evaluating the possibility of hybrid individuals, it is important to consider incomplete linage sorting (ILS) as an explanation. ILS occurs when closely related species have not had enough time to evolve complete genetic divergence from one another. This means that two genetically distinct species can share certain alleles from their common ancestor which can make ILS difficult to distinguish from hybridization. The fact that these presumed hybrid individuals are occurring only in areas of the two overlapping species points to the possibility that incomplete lineage sorting is not the reason for the occurrence of these admixed individuals and they are in fact hybrids. Two of the admixture individuals found in Beaver County were ~23.5 km apart. These results indicate a wide contact zone in this region and there could be either multiple hybrid zones or a wide hybrid zone along the boundary of the two species. Mitochondrial RFLP data identified each admixture individual as *G. bursarius*, meaning the parental cross was between a female *G. bursarius* and a male *G. jugossicularis*.

Genetic diversity within and among Oklahoma and Texas gophers

Heterozygosity is an indicator of genetic diversity within populations (Reed and Frankham 2000). The loss of genetic diversity can be related to inbreeding which can reduce reproductive fitness. Therefore, there is a direct correlation between

heterozygosity and population fitness (Reed and Frankham 2000). When looking at all samples within this project, the PIC value was relatively high (0.823) indicating that there are high levels of genetic diversity within each locus and within each species. The observed heterozygosity (mean HObs = 0.521) was lower than the expected heterozygosity (mean HExp = 0.851) across all loci. Nevo et al. (1974) found that species of *Thomomys* had low heterozygosity (*H*) among populations when examining allozymes. Heterozygosity ranged from 5.6% to 18.4%, with an average *H* of 10.6%. Cothran and Zimmerman (1985) found low *H* in *G. bursarius* (5.5%-6.1%) populations in central Oklahoma based on allozymic data. The levels of heterozygosity found in this study were similar to those found in other studies of *Geomys* that utilized the same microsatellite markers (Welborn et al. 2011; Welborn and Light 2014). Fossorial rodents have been reported to have low heterozygosity across a wide range of taxa (Nevo et al. 1974, 1990; Penney and Zimmerman 1976; Cothran and Zimmerman 1985). Nevo et al. (1990) suggested that low heterozygosity in fossorial rodents could be due to being constrained to a constant environment. This may explain the low levels of heterozygosity seen in *Geomys*.

Conclusions

Before the onset of modern genetic analyses, species were identified based on morphological characteristics. In order for taxa to be truly understood, all genetically distinct species must be recognized. Once all species of a genus have been evaluated, it is easier to understand the evolution of the group as a whole. *Geomys* has been problematic with regards to taxonomy due to the cryptic nature of the genus.

The newly elevated species *G. jugossicularis* (Sudman et al. 2006; Genoways et al. 2008; Chambers et al. 2009) has shown that the genus *Geomys* still needs further evaluation with regards to species boundaries, relationships among species, ecological boundaries, and the roles gene flow and selection have in keeping these species genetically distinct from one another. The most recent and extensive study of *G. jugossicularis* was conducted by Genoways et al. (2008), focusing on *Geomys* in Nebraska. Their findings indicated that *G. jugossicularis* is found throughout southwestern Nebraska, and it has been hypothesized to occur as far south as the Oklahoma and Texas panhandles. To date, details on the range of *G. jugossicularis* and boundary lines between closely related species are unresolved. Using *Cytb* RFLP and sequences, and microsatellite markers, I was able to confirm Genoways et al.'s (2008) hypothesis that the range of *G. jugossicularis* does extend into the Oklahoma and Texas panhandles. My research also has shown that there is potential for hybridization between *G. bursarius* and *G. jugossicularis* in this region. Further genetic evaluation needs to be conducted to determine the detailed boundaries of *G. jugossicularis* and *G. bursarius* in the Oklahoma and Texas panhandles, as well as determine the extent of contact zones and hybridization between these two species in this region.

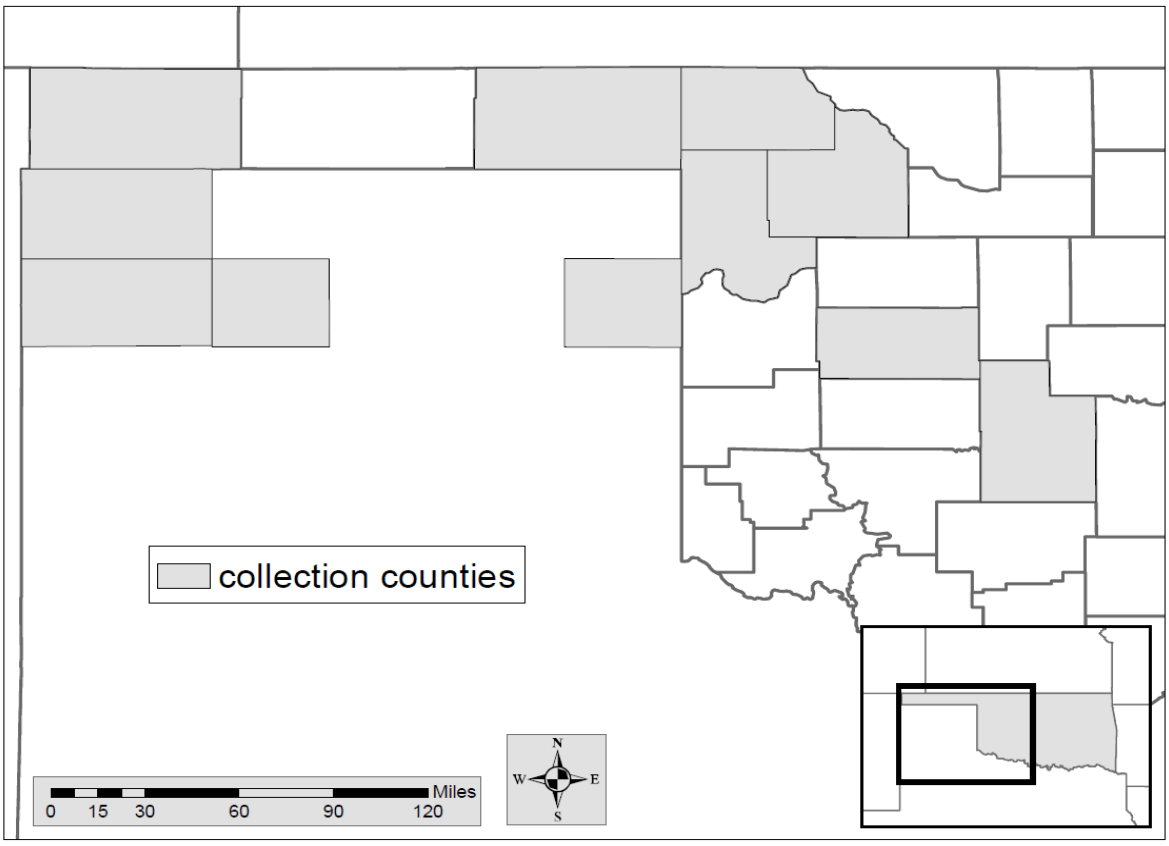

Figure 2.1: Map of the Oklahoma and Texas panhandles showing collection localities (shaded counties). This map was generated using ArcGIS (ESRI 2011).

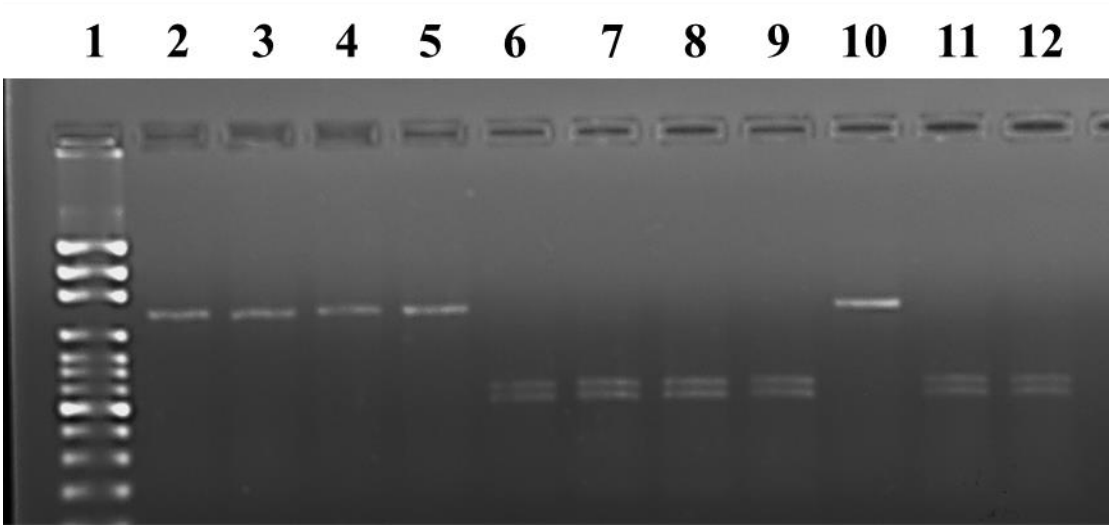

Figure 2.2: An example of electrophoresis results for PCR-RFLP of the *Cytb* gene comparing *G. bursarius* to *G. jugossicularis* using the enzyme BamHI. Lane one contains a 100 bp ladder. Samples were identified as *G. bursarius* if they remained uncut (1140 bp; lanes 2-5, 10). Samples were identified as *G. jugossicularis* if there were two fragments (530 bp and 610 bp; lanes 6-9, 11-12).

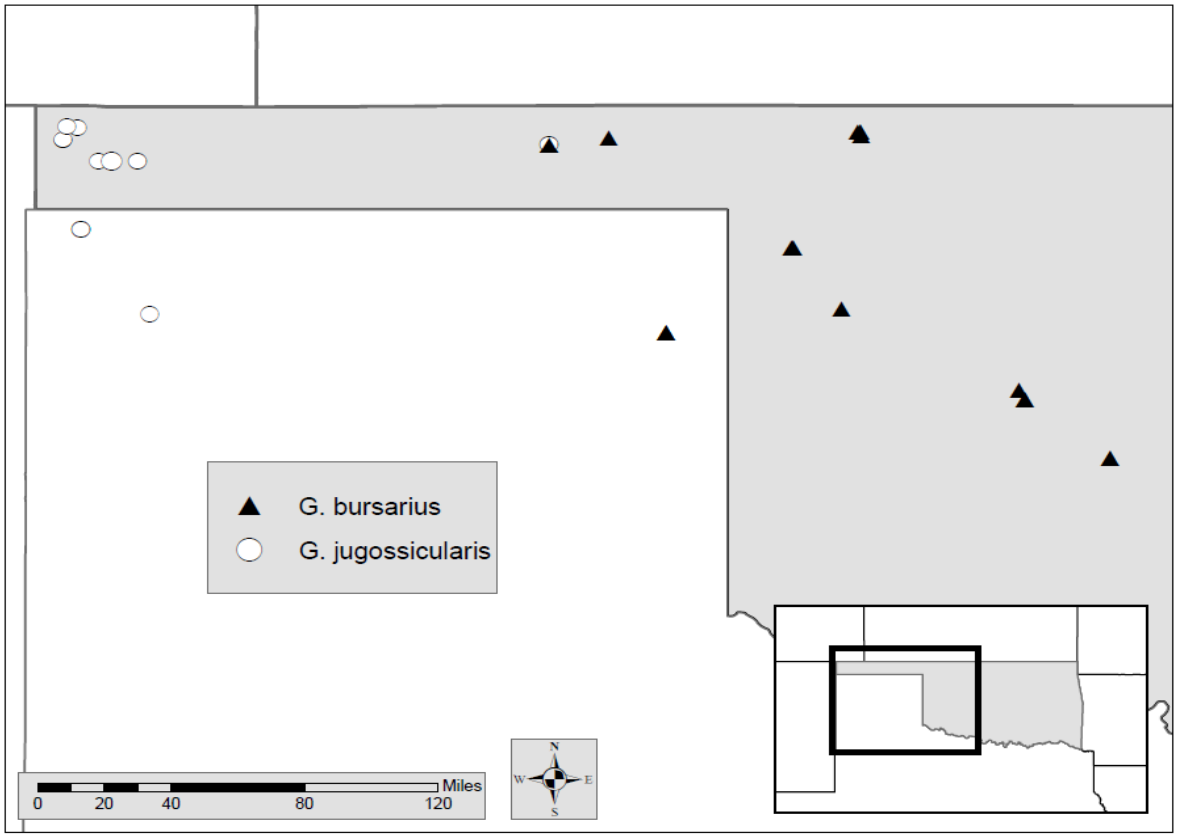

Figure 2.3: Map of geographic locations of samples from the Oklahoma and Texas panhandles based on results of *Cytb* PCR-RFLP identification. Black triangles represent individuals identified as *G. bursarius*, whereas white circles represent individuals identified as *G. jugossicularis.* This map was generated using ArcGIS (ESRI 2011).

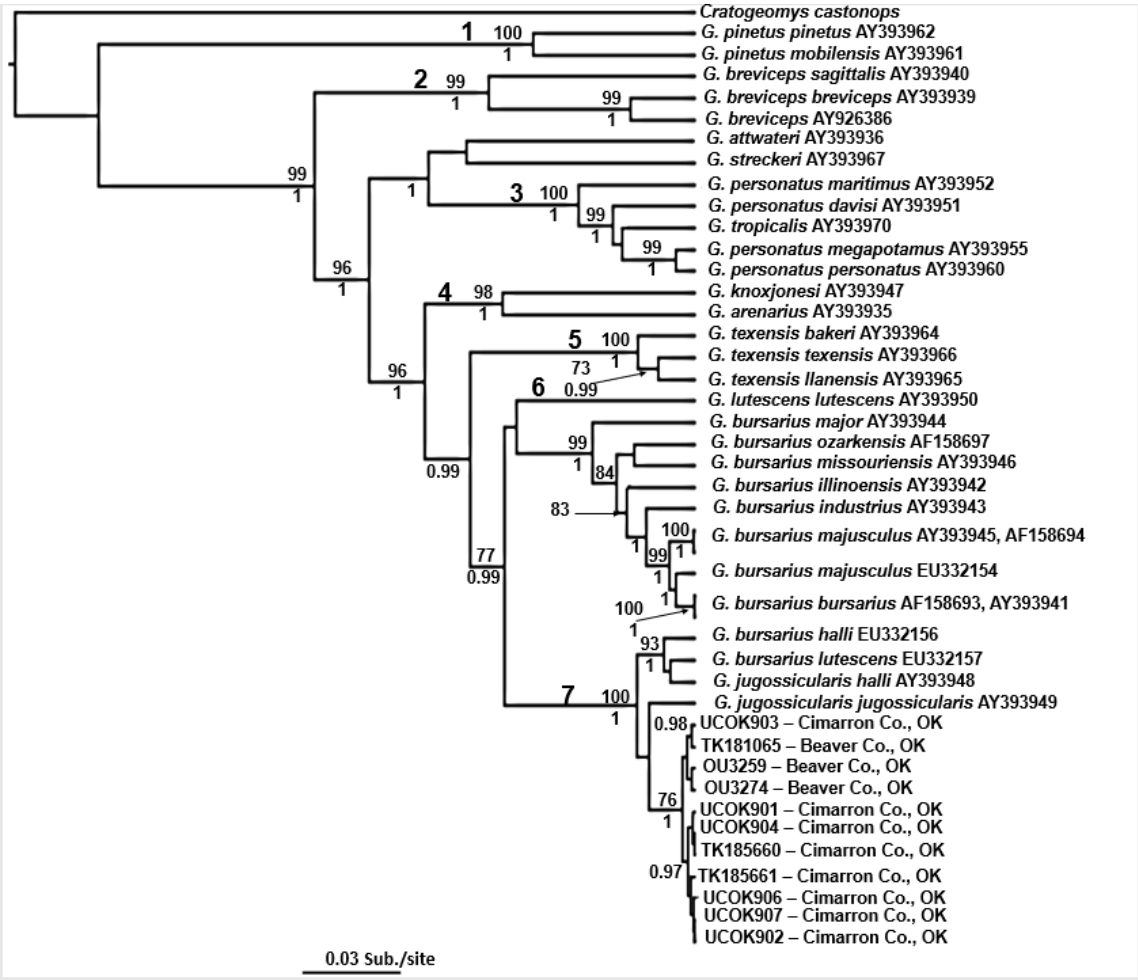

Figure 2.4: Bayesian inference tree containing individuals belonging to *Geomys* species and subspecies. The tree was generated using BEAST 1.8.2 (Drummond et al. 2012) and the HKY+I+G model. *Cratogeomys* was used as the outgroup taxon. Eleven samples from the Oklahoma panhandle were collected and sequenced for this project, the remaining 31 sequences were obtained from GenBank. Bootstrap values are shown above the branches and posterior probabilities below the branches. Bootstrap values were generated from a maximum likelihood tree produced using MEGA 7 (Kumar et al. 2016).

Figure 2.5: Barplot depicting clustering results from the Structure run consisting of all gopher samples from the Oklahoma and Texas panhandles (n = 54). Each bar on the figure represents an individual. Colors correspond to the map in Fig. 2.6.

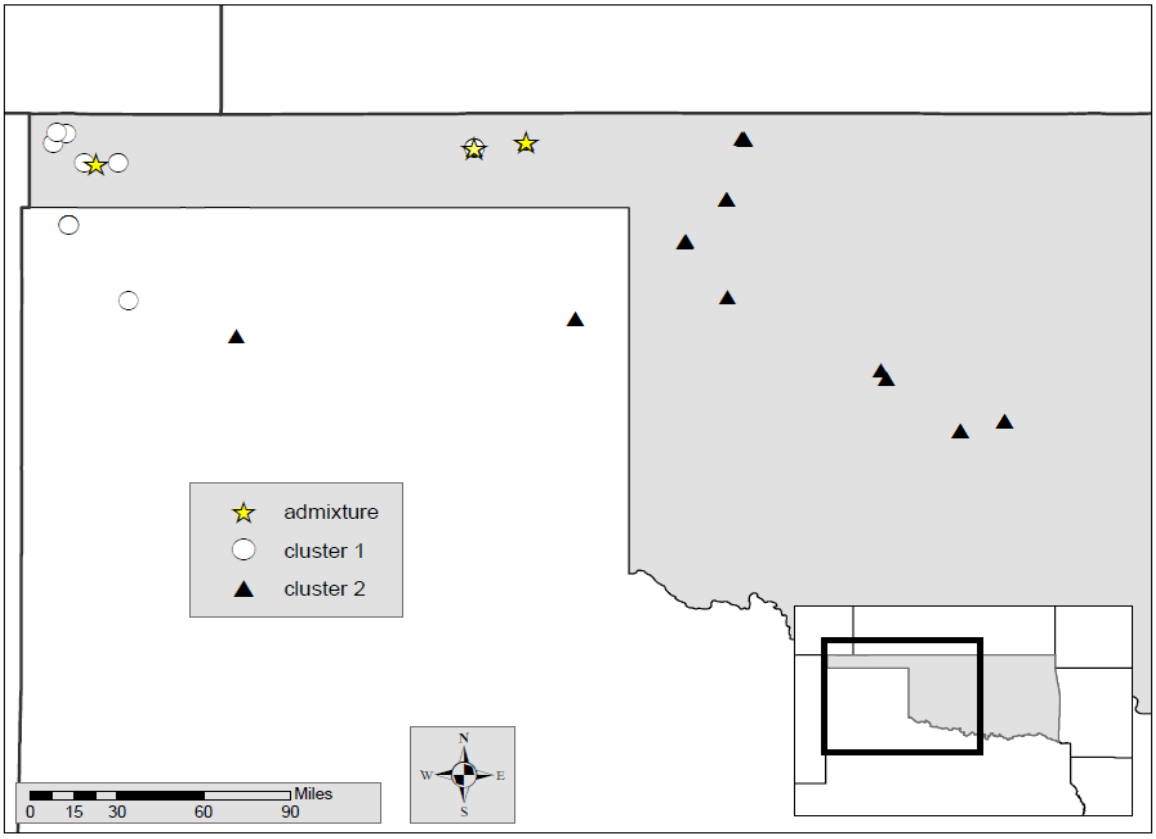

Figure 2.6: A map of cluster results from the Structure run consisting of all Oklahoma and Texas panhandle samples. Black triangles correspond to the black lines in Fig. 2.5 and most likely represent *Geomys bursarius* samples. White circles correspond to the white lines in Fig. 2.5 and most likely represent *G. jugossicularis* individuals. Stars indicate admixture individuals. Admixture was determined based on assignment to a given cluster with a confidence level <80%. This map was generated using ArcGIS (ESRI 2011).

Table 2.1: Mitochondrial DNA cytochrome-*b* gene primers used for this study. The name, sequence, type, and source are provided for each primer.

Primer Name	Primer Sequence	Primer Type	Source
H15915	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	Amplification	Irwin et al. 1991
L14735	TGA AAA ACC ATC GTT GTT AAT TCA ACT	Amplification	Elrod et al. 2000
MVZ05	CGA AGC TTG ATA TGA AAA ACC ATC GTT G	Sequencing	Smith and Patton 1993
H15906	CAT CTC CGG TTT ACA AGA CCT AAG TAA T	Sequencing	Elrod et al. 2000
400F	CCA TGA GGA CAA ATA TCC TTC TGA GGG	Sequencing	Edwards et al. 2001
400R	GCC CTC AGA AGG ATA TTG TCC CAT GG	Sequencing	Peppers and Bradley 2000
700L	CCC CAG CAC ATA TTA AAC CAG AAT G	Sequencing	Peppers and Bradley 2000
L15049	GCC TGT ACA TCC ACA TCG GAC GAG G	Sequencing	Irwin et al. 1991

Table 2.2: Microsatellite markers used in this study. The primer name, sequences (F = forward, R = reverse), size in base pairs (bp), annealing temperatures, and source are listed for each pair.

Primer Name	Primer Sequence	Size (bp)	Annealing Temperatures	Source
Gbr09	F- TGGCTCAAGTGAGAGCATCA	214	58°C, 56°C, 52°C	Welborn et al. 2011
	R- GGAGGAGGAACAAGCAATCA			
Gbr10	F- TAGTGCATGCTCTGGCTTTG	235	57°C, 55°C, 51°C	Welborn et al. 2011
	R- AAATGCCCTCCAGAAGGAAC			
Gbr15	F- CTCTCCCTCAGCTCAGCAGT	212	58°C, 56°C, 52°C	Welborn et al. 2011
	R- GTGTCCAGCCCAGTTATGCT			
Gbr25	F- CCTGGGAGACTAGCATGAGG	227	58°C, 56°C, 52°C	Welborn et al. 2011
	R- CACAAGAAAGCCAGAAGTGC			
Gbr27	F- TGATGACACGCTGACTTTCC	229	58°C, 56°C, 52°C	Welborn et al. 2011
	R- TGGAGGTGTAGCTCAAGTGG			
Gbr33	F- GTGGTAGTGGTGGTGTTTGC	227	56°C, 54°C, 50°C	Welborn et al. 2011
	R- ACACTGGAGTGTCTCATGTGG			
Tm1	F- TCACATACTAGCCCAAAGTCCTC	181	58°C, 56°C, 52°C	Steinberg 1999
	R- GTGGTAGAGCAAAAGAAGCTGAA			
Tm2	F- CCGGATCTTGGATTAGGCAT	173	58°C, 56°C, 52°C	Steinberg 1999
	R-GGCTGTTTTAATTTCCTTCATGT			
Tm7	F- TCTACTGAACCACCAGAAAATCAA	288	58°C, 56°C, 52°C	Steinberg 1999
	R- AGCACTGGACTTGAACACAAATAC			

Table 2.3: Selected pairwise distance values comparing individuals from GenBank to samples collected from within the Oklahoma panhandle. Pairwise distance values were generated using the TN93 model in MEGA 7 (Kumar et al. 2016).

Comparison	**Mean Distance Value (%)**
Within OK panhandle samples	0.2 (range 0-0.6)
G. j. jugossicularis – OK panhandle	1.9 (range 1.8-2)
G. b. lutescens – G. j. halli	1.4
G. b. halli – G. b. lutescens/G. j. halli	1.5 (range 1.1-1.8)
G. j. jugossicularis – G. b. halli/G. b. lutescens/G. j. halli	2.3 (range 2.1-2.4)
G. b. major – OK panhandle samples	5.9 (range 5.9-6)

Table 2.4: CERVUS (Marshall et al. 1998) results for *Geomys* samples collected in the Oklahoma and Texas panhandles. Separate analyses were run for all sample (*n* = 54), only *G. jugossicularis* samples (*n* = 19), and only *G. bursarius* samples (*n* = 35). Observed heterozygosity (HObs), expected heterozygosity (HExp), polymorphic information content (PIC), and number of alleles per locus are provided for all loci.

Locus	HObs	HExp	PIC	Alleles per locus
All Samples				
Gbr09	0.741	0.878	0.859	15
Gbr10	0.608	0.919	0.903	16
Gbr15	0.519	0.787	0.762	14
Gbr25	0.774	0.927	0.913	17
Gbr27	0.189	0.596	0.504	4
Gbr33	0.413	0.895	0.875	13
Tm1	0.373	0.893	0.874	15
Tm6	0.370	0.823	0.792	12
Tm7	0.704	0.938	0.925	20
Average	0.521	0.851	0.823	14.000
G. jugossicularis				
Gbr09	0.684	0.856	0.815	9
Gbr10	0.563	0.867	0.821	9
Gbr15	0.158	0.154	0.146	4
Gbr25	0.833	0.898	0.861	13
Gbr27	0.333	0.286	0.239	2
Gbr33	0.188	0.841	0.789	6
Tm1	0.294	0.783	0.723	6
Tm6	0.474	0.494	0.432	4
Tm7	0.737	0.926	0.893	14
Average	0.474	0.678	0.635	7.444
G. bursarius				
Gbr09	0.743	0.876	0.850	14
Gbr10	0.657	0.923	0.903	16
Gbr15	0.714	0.885	0.861	14
Gbr25	0.800	0.921	0.901	17
Gbr27	0.143	0.493	0.429	4
Gbr33	0.500	0.892	0.865	12
Tm1	0.382	0.914	0.892	13
Tm6	0.371	0.805	0.767	10
Tm7	0.657	0.925	0.905	19
Average	0.552	0.848	0.819	13.222

Appendix 2.1: Table representing all *Geomys* samples used in this study and their collection sites or museum sources. RFLP ID results and Structure clustering results are listed for each individual. OU = Sam Noble Museum of Natural History; TTU = NSRL, The Museum of Texas Tech University; MSB = Museum of Southwestern Biology; UCOK = University of Central Oklahoma Natural History Museum (these samples were collected for this project). * = a putative hybrid individual based on conflicting results from *Cytb* and microsatellite analyses. N/A = data not available.

Sample ID	Museum Source	State	County	Latitude	Longitude	RFLP ID	Structure ID
OU3225	OU	OK	Beaver	36.8109	-100.7752	*bursarius*	*bursarius*
OU3252	OU	OK	Beaver	36.8109	-100.7752	N/A	*bursarius*
OU3259	OU	OK	Beaver	36.8109	-100.7752	*jugossicularis*	admixture
OU3260	OU	OK	Beaver	36.8109	-100.7752	*bursarius*	**jugossicularis*
OU3274	OU	OK	Beaver	36.8109	-100.7752	*jugossicularis*	*jugossicularis*
OU4275	OU	OK	Beaver	36.8109	-100.7752	*jugossicularis*	*jugossicularis*
OU9423	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9424	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9425	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9426	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9440	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
TK26732	TTU	TX	Moore	35.8229	-101.9689	N/A	*bursarius*
TK28873	TTU	TX	Hartley	36.0054	-102.5078	*jugossicularis*	*jugossicularis*
TK52423	TTU	TX	Hemphill	35.9143	-100.2684	*bursarius*	*bursarius*
TK52481	TTU	TX	Hemphill	35.9143	-100.2684	*bursarius*	*bursarius*
TK52482	TTU	TX	Hemphill	35.9143	-100.2684	*bursarius*	*bursarius*
TK181065	TTU	OK	Beaver	36.8431	-100.5154	*bursarius*	*bursarius*
TK181066	TTU	OK	Beaver	36.8431	-100.5154	*bursarius*	admixture
TK185660	TTU	OK	Cimarron	36.7335	-102.7287	*jugossicularis*	*jugossicularis*
TK185661	TTU	OK	Cimarron	36.7323	-102.6707	*jugossicularis*	admixture
TK197205	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197207	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197204	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197190	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197201	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197202	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
TK197206	TTU	TX	Dallam	36.4089	-102.8062	*jugossicularis*	*jugossicularis*
MSB273626	MSB	OK	Caddo			N/A	*bursarius*
MSB273628	MSB	OK	Caddo			N/A	*bursarius*
MSB273629	MSB	OK	Caddo			N/A	*bursarius*
MSB273630	MSB	OK	Caddo			*bursarius*	*bursarius*
MSB273652	MSB	OK	Caddo			*bursarius*	*bursarius*

UCOK901	UCO	OK	Cimarron	36.8397	-102.8828	*jugossicularis*	*jugossicularis*
UCOK902	UCO	OK	Cimarron	36.8397	-102.8828	*jugossicularis*	*jugossicularis*
UCOK903	UCO	OK	Cimarron	36.8912	-102.8214	*jugossicularis*	*jugossicularis*
UCOK904	UCO	OK	Cimarron	36.8985	-102.8670	*jugossicularis*	*jugossicularis*
UCOK905	UCO	OK	Ellis	36.0283	-99.5065	*bursarius*	*bursarius*
UCOK906	UCO	OK	Cimarron	35.4787	-97.2170	*jugossicularis*	*jugossicularis*
UCOK907	UCO	OK	Cimarron	36.7333	-102.5590	*jugossicularis*	*jugossicularis*
UCOK983	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK984	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK985	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK986	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK987	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK993	UCO	OK	Custer	35.5950	-98.7107	*bursarius*	*bursarius*
UCOK994	UCO	OK	Custer	35.5950	-98.7104	*bursarius*	*bursarius*
UCOK995	UCO	OK	Custer	35.6384	-98.7380	*bursarius*	*bursarius*
UCOK996	UCO	OK	Harper	36.8585	-99.4209	*bursarius*	*bursarius*
UCOK997	UCO	OK	Harper	36.8704	-99.4248	*bursarius*	*bursarius*
UCOK998	UCO	OK	Harper	36.8704	-99.4248	*bursarius*	*bursarius*
UCOK999	UCO	OK	Harper	36.8705	-99.4357	*bursarius*	*bursarius*
UCOK1000	UCO	OK	Ellis	36.3227	-99.7155	*bursarius*	*bursarius*
UCOK1001	UCO	OK	Ellis	36.3215	-99.7170	*bursarius*	*bursarius*
UCOK1002	UCO	OK	Ellis	36.3193	-99.7221	*bursarius*	*bursarius*

Appendix 2.2: Table representing *Geomys* GeneBank samples used in this study. Accession number and species identification are listed for each individual.

Accession #	**Species ID**
AY393962	G. pinetus pinetus
AY393961	G. pinetus mobilensis
AY393940	G. breviceps sagittalis
AY393939	G. breviceps breviceps
AY926386	G. breviceps
AY393936	G. attwateri
AY393967	G. streckeri
AY393952	G. personatus maritimus
AY393951	G. personatus davisi
AY393970	G. tropicalis
AY393955	G. personatus megapotamus
AY393960	G. personatus personatus
AY393947	G. knoxjonesi
AY393935	G. arenarius
AY393964	G. texensis bakeri
AY393966	G. texansis texensis
AY393965	G. texensis llanesis
AY393950	G. lutescens lutescens
AY393944	G. busarius major
AF158697	G. bursarius ozarkensis
A393946	G. bursarius missouriensis
AY393942	G. bursarius illinoensis
AY393943	G. bursarius industrius
AF393945	G. bursarius majusculus
AF158694	G. bursarius majusculus
EU332154	G. bursarius majusculus
AF158693	G. bursarius bursarius
AY393941	G. bursarius bursarius
EU332156	G. bursarius hali
EU332157	G. bursarius lutescens
AY393948	G. jugossicularis hali
AY393949	G. jugossicularis jugossicularis

CHAPTER 3

EVALUATION OF BOUNDARY LINES, CONTACT ZONES, AND HYBRIDIZATION IN CENTRAL OKLAHOMA POCKET GOPHERS

INTRODUCTION

Because pocket gophers depend heavily on soil types, have low mobility, and isolated populations, all of which act to restrict their distribution, they are an ideal study organism for understanding divergence and speciation mechanisms. Currently, there are 11 recognized species of *Geomys* pocket gophers that occur throughout the central plains of the United States (Sudman et al. 2006). There is little morphological variation between species, which makes it difficult to readily identify individuals to the species level (Mauk et al. 1999). The lack of morphological differences between *Geomys* species has led them to be classified as a cryptic species (Sulentich et al. 1991; Mauk et al. 1999; Sudman et al. 2006).

Geomys form contact zones in a number of areas across their range (e.g., Baker et al. 1989). In some situations where overlapping species occur, hybridization has been reported (Cothran and Zimmerman 1985; Baker et al. 1989; Genoways et al. 2008). The potential for hybridization along these contact zones further complicates species identification. Pembleton and Baker (1978) examined a hybrid zone between subspecies of *G. bursarius* (plains pocket gopher) now considered two genetically distinct species, *G. bursarius* and *G. knoxjonesi* (Knox Jones's pocket gopher), in eastern New Mexico and found F_1 hybrid offspring with the potential of F_2 offspring. Baker et al. (1989) used mitochondrial and ribosomal DNA to identify 41 admixture individuals in the same

hybrid zone. The hybrid offspring were shown to have reduced fitness, although backcrossing did occur with both parental types and reproduction occurred between highly backcrossed individuals. The study of hybrid zones between *Geomys* species has resulted in important findings aiding in the understanding of hybrid zone formation and maintenance, and various evolutionary processes involved in speciation mechanisms.

Oklahoma pocket gophers

It was not until recently that *G. breviceps* (Baird's pocket gopher) was determined to be genetically separate from *G. bursarius*. Bohlin and Zimmerman (1982) conducted a study covering a wide range of *G. bursarius* from 37 populations throughout Texas, Oklahoma, and Louisiana, using electrophoretic variation in proteins. They concluded that there were two distinct isolated gene pools in Oklahoma between what were then considered two chromosomal races of *G. bursarius*, and recommended that they be recognized as separate species. Currently, three species of *Geomys* are known to occur in Oklahoma: *G. bursarius* in western Oklahoma, *G. breviceps* in eastern Oklahoma, and a third recently described species of pocket gopher, *G. jugossicularis* (Hall's pocket gopher), in the Oklahoma panhandle (Chapter 2).

Because Oklahoma pocket gophers are cryptic species the boundary between *G. bursarius* and *G. breviceps*, which meets in central Oklahoma, is difficult to define. The most extensive study of pocket gophers performed in Oklahoma was by Heaney and Timm (1983). Their study consisted of 1,400 pocket gophers collected throughout the central plains spanning across numerous states. This study included cranial measurements and other morphological characteristics. Discriminant function analyses of cranial

measurements and body size comparison indicated *Geomys* species collected from a large geographical range were indistinguishable from one another. They also concluded that gophers collected from southeastern Oklahoma were indistinguishable from those in eastern Oklahoma and distinct from those in central Oklahoma (Heaney and Timm 1983). Using morphological data, Heaney and Timm (1983) proposed that the boundary line between *G. bursarius* and *G. breviceps* runs north to south in central Oklahoma (Fig. 3.1). This boundary roughly coincides with the Interstate 35 corridor. Even though the characters used by Heaney and Timm (1983) to group the samples were quantitative, the descriptions and identifying characteristics of the species were qualitative and hard to assess on individual samples, thus the need for molecular characters to identify specimens.

Heaney and Timm (1983) summarized putative contact zones between the two species of pocket gophers and suggested the possibility of hybridization in central Oklahoma. Bohlin and Zimmerman (1982) and Cothran and Zimmerman (1985) established the boundary line and identified a zone of contact between the two species in Norman, Oklahoma. Five putatively hybridizing populations were examined and one F_1 offspring was found. The remaining hybrid individuals were F_2's and showed backcrosses with both parental species. Both Heaney and Timm (1983) and Bohlin and Zimmerman (1982) suggested possible isolation of the two species as a result of soil/habitat type. Additionally, Heaney and Timm's (1983) findings suggested a narrow contact zone.

The objectives of my research were to use molecular markers to 1) determine if the boundary between *G. bursarius* and *G. breviceps* is in central Oklahoma, as proposed

by Heaney and Timm (1983; Fig. 3.1) based on morphological data, was supported by molecular markers, 2) determine the location of contact zones between the species in central Oklahoma, 3) determine if hybridization occurred in areas where the two species come into contact, and 4) assess genetic diversity within species and genetic differentiation between species found in central Oklahoma. Based on the results of Heaney and Timm (1983), I expected the zones of contact between the two species to be narrow (≤ 8 km).

METHODOLOGY

Sample collection

I conducted trapping from fall of 2015 to summer of 2017. As discussed in Chapter 2, *G. jugossicularis* was determined to occur in the Oklahoma panhandle. The presence of a third species in Oklahoma was not the focus of this research, therefore Oklahoma panhandle samples were not included in this project. To ensure geographic variation within each species (*G. bursarius* and *G. breviceps*) was well represented, 118 samples were collected or obtained through museum loans from 23 counties (Fig. 3.2; Appendix 3.1). A majority of the collection sites were located in central Oklahoma where the presumed boundary line is located. Trapping localities were based on collection sites reported by Heaney and Timm (1983). I also collected samples within the known geographic range of each species (western Oklahoma – *G. bursarius*, eastern Oklahoma – *G. breviceps*). When available, I obtained tissue loans from various institutes (Appendix 3.1). I collected specimens using Victor and Macabee gopher kill traps, which are

approved by the American Society of Mammologists (ASM) IACUC (Sikes et al. 2016). The University of Central Oklahoma (UCO) IACUC (#14011) has approved the described protocol. Specimens were collected from both public and private properties. All trapping done on private property was conducted with landowner's permission. Upon capture, I assigned each specimen an identification number and geographic coordinates in the form of latitude and longitude using a handheld GPS. Specimens were immediately stored on ice before being transported to the lab for processing. During processing, I recorded standard measurements and sex for each specimen, and collected kidney, heart, liver, lung, leg muscle, spleen, and colon tissues. All specimens and tissue samples were deposited in the UCO Natural History Museum (Appendix 3.1).

DNA extraction, PCR amplification, and genotyping

I extracted DNA from liver samples using DNeasy Blood and Tissue Extraction kits (Qiagen). I measured nucleic acid concentrations with the NanoDrop 2000 (ThermoFisher Scientific) to ensure high quality, intact DNA was being used for all genetic analyses. The S100 Thermal Cycler (Bio-Rad) was used to perform polymerase chain reactions (PCR).

I amplified the complete mitochondrial DNA cytochrome-*b* (*Cytb*) gene (1140 bp) using two primers, L14735 and H15906 (Elrod et al. 2000; Table 3.1), for all 118 individuals. The PCR reaction volume was 25 µL, which consisted of 12.5 µL nuclease free water, 8.3 µL Failsafe premix C (Epicentre), 1.0 µL of each amplification primer, 0.2 µL GoTaq Flexi DNA polymerase (Promega), and 2 µL of DNA. PCR thermal cycling parameters were as follows: initial denaturation at 94°C for 2 minutes; followed by 38

cycles of 92°C denaturation for 15 seconds, 54°C annealing for 1 minute, and 72°C extension for 1 minute and 10 seconds; and a final 72°C extension for 10 minutes.

I used nine microsatellite primer pairs (Table 3.2) to genotype all 118 samples of *Geomys* collected in Oklahoma. Welborn et al. (2011) developed six of the nine primers specifically to amplify loci for *G. breviceps*, and these primers have been shown to successfully amplify DNA of other *Geomys* species. The remaining three primers were developed for *Thomomys* (Steinberg 1999) and have been shown to amplify DNA of *Geomys* (Welborn et al. 2011). I performed PCR amplifications in 25 µL reaction volumes containing 14.3 µL nuclease free water, 5 µL 5X colorless GoTaq buffer (Promega), 2 µL $MgCl_2$ (Promega), 0.75 µL 10 mM mixture of dNTPS (New England Biolabs), 0.6 µL of each primer, 0.25 µL GoTaq Flexi DNA polymerase, and 1.5 µL DNA. Annealing temperatures differed across multiple loci and can be found in Table 3.2. The general PCR thermal cycling parameters were as follows: initial denaturation at 95°C for 3 minutes; followed by 9 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, and 72°C extension for 1 minute; 9 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, and 72°C extension for 1 minute; 14 cycles of 95°C denaturation for 30 seconds, appropriate annealing temperature for 45 seconds, 72°C extension for 1 minute; and a final 72°C extension for 10 minutes. I analyzed amplified microsatellite PCR products using an ABI3130 or ABI3500 Genetic Analyzer (ThermoFisher Scientific). I prepared the samples for the genetic analyzer using 9.25 µL Hi-Di formamide (ThermoFisher Scientific), 0.25 µL size standard (Genescan 500 ROX; ThermoFisher Scientific), and 0.5 µL PCR product. I visualized, edited, and scored microsatellite genotypes using

GeneMapper Software 5 (ThermoFisher Scientific). The use of nine primer pairs provided an optimal genetic profile for each sample which allowed for precise individual identification.

Cytochrome-b data analyses

I used restriction fragment length polymorphism (RFLP) of the *Cytb* gene to determine species identification of pocket gophers collected in Oklahoma. Restriction enzymes, also known as restriction endonucleases, are enzymes that cut DNA at specific regions (Wolf et al. 1999). Variation in restriction enzyme cut sites between species allows for a simple and rapid means of species identification (Avise et al. 1979; Baker et al. 1989). I used the EcoRV (New England Biolabs) restriction enzyme to differentiate *G. bursarius* from *G. breviceps*. This enzyme does not cut in *G. bursarius* samples, resulting in a fragment of 1140 base pairs (bp), which is the total length of the *Cytb* gene in this species. *G. breviceps* has one cut site, resulting in two restriction fragments that were 413 bp and 727 bp in length.

Once samples were amplified, the EcoRV enzyme was used to digest samples. The manufacturer's protocol was followed and called for 3 µL of PCR product, 5 µL of 10X NEbuffer, 1 µL enzyme, and 41 µL of nuclease free water for a total reaction volume of 50 µL. I incubated samples for 1 hour at 37°C, then performed gel electrophoresis on a 2% agarose gel using 0.5X TBE (Tris/Borate/EDTA) buffer. Gels were set to 120 volts and run until the samples migrated halfway down the gel. I viewed the gels using the UVP GelDoc-It 310 Imaging System (ThermoFisher Scientific). I

visually assigned each sample a species identification based on the number and length of fragments.

Microsatellite data analyses

Because mtDNA is maternally inherited and cannot be used to detect hybrids, I used nuclear DNA to identify admixture individuals. Microsatellites also can be used to separate samples into distinct genetic clusters. Structure 2.3.4 (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009) was used to identify admixture individuals and estimate distinct genetic clusters represented by the Oklahoma samples (n = 118). This program infers the presence of genetically distinct groups and estimates the number of clusters occurring in the represented samples using allele frequency data. Parameters for the Structure run included the Correlated Allele frequency model, 100,000 burn-in steps, 1,000,000 sampling steps, and 5 iterations for each K (number of putative populations tested). K was set at 1 - 5. The results obtained from Structure were uploaded to Structure Harvester (Earl and vonHoldt 2012) to determine the most likely value of K that best fit the data based on the Evanno et al. (2005) method. Individuals were assigned to clusters based on an 80% threshold. Individuals that did not fall within the 80% threshold were considered admixture individuals. For example, an individual sharing at least 20.9% of their alleles with one cluster and the remaining 79.9% with another cluster was considered an admixture.

The results of a cluster analysis are presented as a matrix where each individual in the dataset is assigned a likelihood value of belonging to each cluster provided the allelic frequencies of the complete dataset. Individuals are assigned to separate clusters as

interpreted by the likelihood values. Because the Structure analysis was set to five iterations, there were a total of five results groups, all of which were similar. The Cluster Matching and Permutation Program (CLUMPP) v1.1.2 (Jakobsson and Rosenberg 2007) was used to average all individuals across the independent runs for each value of K, using the FullSearch option. Barplots were created using distruct v1.1 (Rosenberg 2004). This program allows the user to adjust the barplot color and size for better interpretation.

To make sure there were no genotypic scoring errors before additional analyses were conducted, I used MICROCHECKER v2.2.1 (Van Oosterhout et al. 2004) to determine if there were null alleles, large-allele dropout, or stutter-induced typing errors at each locus. This program randomizes the observed alleles for each locus in each individual sample to construct random genotypes. These random genotypes were then compared to the observed genotypes to determine the frequency of allele-specific homozygote size classes and allele-specific heterozygote size classes. The cumulative binomial distribution (Weir 1996) was used to determine the probability of both classes. The observed classes were then compared to the probability values to determine if there were any genotyping errors. Because this project involves multiple species, the data was divided and analyzed three separate ways: all samples collected throughout the entire project (n = 118), only samples identified as *G. bursarius* (n = 67), and only samples identified as *G. breviceps* (n = 51). These runs were conducted including the missing data values.

I used the program FSTAT 2.9.3.2 (Goudet 1995, 2001) to assess the data for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium, as well as to estimate allelic diversity and F_{st}. The data was divided into three datasets as

previously described and admixture individuals were removed for a clearer interpretation of the results. Dataset one (n = 114) excluded four admixture individuals and the rest were assigned to two separate populations (*G. bursarius* and *G. breviceps*) based on RFLP and clustering results. Dataset two contained only samples identified as *G. bursarius* (n = 66), excluding one admixture sample. Dataset three contained only samples identified as *G. breviceps* (n = 48), excluding three admixture individuals. Hardy-Weinberg was tested over all samples and population differentiation did not assume HWE. Hardy-Weinberg tests were performed per locus and sample. A pairwise test of differentiation also was performed in FSTAT. Genotypic equilibrium was tested between all pairs of loci, and a 5/100 nominal level for multiple tests was used. The program CERVUS 3.0.7 (Marshall et al. 1998; Slate et al. 2000; Kalinowski et al. 2007, 2010) was used to estimate observed and expected heterozygosity, polymorphic information content (PIC), as well as perform allelic frequency analyses. Samples for CERVUS analyses were divided into three datasets as previously discussed.

Once all individuals were assigned to a putative species based on PCR-RFLP and Structure, ArcGIS (ESRI 2011) was used to create maps of both sets of results. The dataset included all individuals and their locations (longitude and latitude; Appendix 3.1), as well as their species ID or the cluster to which they were assigned. A single point feature class was created for the geodatabase, the NAD 1983 coordinates system was used, and the United States Census Bureau cartographic boundary shapefile was used.

RESULTS

RFLP analysis

Ninety-four percent (112 of 118) of the samples were analyzed successfully using PCR-RFLP of the *Cytb* gene. The digestion of the *Cytb* gene using the enzyme EcoRV created a unique RFLP profile between *G. bursarius* and *G. breviceps* samples (Fig. 3.3). There were 64 individuals identified as *G. bursarius* and 48 individuals identified as *G. breviceps*. Six individuals were unable to be identified successfully using this protocol. These samples included UCOK927 (Oklahoma Co.); UCOK 934-935 (Logan Co.); and MSB273626, 273627, and 273629 (Caddo Co.; Appendix 3.1). The results for the RFLP analysis were mapped using ArcGIS (Fig. 3.4). It should be noted that there were two individuals identified as *G. bursarius* (TK27149, Hughes Co., and UCOK977, McCurtain Co.; Appendix 3.1) that were found in eastern Oklahoma.

Genetic structure

Structure Harvester was used to interpret the Structure results and, based on the data, I concluded that K = 2, meaning there were two genetically distinct clusters occurring throughout Oklahoma, excluding the panhandle. The F_{st} value, estimated in Fstat, was 0.129, indicating moderate genetic divergence between the groups (Wright 1978). The results for the cluster analyses are shown in Figure 3.5. There were four admixture individuals identified. Two admixture individuals were found in Seminole County and the remaining two admixture individuals were collected from Logan and Marshall Counties (Appendix 3.1). Excluding admixture individuals, 66 individuals were assigned to cluster one and 48 individuals were assigned to cluster two. The clustering results where then mapped using ArcGIS (Fig. 3.6). Again, two individuals identified as

G. bursarius (TK27149, Hughes Co., and UCOK977, McCurtain Co.; Appendix 3.1) were found in eastern Oklahoma.

Genotyping errors

The MICROCHECKER run containing all individuals showed low observed heterozygosity across all loci except Gbr09, which could be a possible indicator of null alleles. There was no evidence of high allele dropout, but possible stuttering may have resulted in scoring errors in loci Gbr10, Gbr27, Tm1, Tm6, and Tm7. The dataset containing only individuals identified as *G. bursarius* showed low heterozygosity across all loci with the exception of Gbr09. There was no evidence of high allele dropout, but there was possible error due to stuttering at loci Gbr10, Gbr27, and Tm7. The dataset containing only individuals identified as *G. breviceps* was shown to have lower than expected heterozygosity among all loci except Gbr09. There was no large allele dropout among the loci, but possible error could have occurred due to stuttering at loci Grb10, Tm1, Tm6, and Tm7. All samples were scored twice before any additional analyses to ensure no user error in scoring or errors due to stuttering. The loci were in HWE (adjusted p-value = 0.00278). The adjusted p-value for genotypic disequilibrium was 0.01389. There were no pairs of loci that were found to be in linkage disequilibrium. However, Gbr09 x Tm7, Tm1 x Tm7, and Gbr10 x Gbr15 were on the threshold of linkage disequilibrium. The value for these pairs was 0.00139. Because this value was greater than or equal to the adjusted p-value, the loci pairs were considered not to be in linkage disequilibrium.

Genetic diversity

For the overall dataset, observed heterozygosity (HObs) was lower across all loci when compared to the expected value (mean HObs = 0.543; Table 3.3). The mean number of alleles per locus was 16.333, the mean expected heterozygosity was 0.849, and the mean PIC value was 0.829. Using the Fstat output file, unique alleles were identified within the two species in Oklahoma examined in this study. The four admixture samples were excluded and all loci were examined in this analysis. At locus Gbr09, nine unique alleles were identified for *G. bursarius*. At locus Gbr10, there were six unique alleles for *G. bursarius*. Locus Gbr15 contained six unique alleles for *G. bursarius*. For locus Gbr25, *G. breviceps* had one unique allele and *G. bursarius* contained eight unique alleles. At locus Gbr27, there was one unique allele found for *G. bursarius*. For locus Gbr33, *G. bursarius* had six unique alleles. At locus Tm1, *G. breviceps* had two unique alleles and *G. bursarius* had eight unique alleles. At locus Tm6, *G. bursarius* had 8 unique alleles. For locus Tm7, *G. bursarius* had seven unique alleles. Genetic diversity values for *G. bursarius* and *G. breviceps* are provided in Table 3.3.

DISCUSSION

Species identification

Between RFLP analysis of the *Cytb* gene and Structure analysis of nine microsatellite loci, I was able to successfully identify morphologically cryptic gophers in central Oklahoma. Based on RFLP results, 64 individuals had the expected banding pattern of *G. bursarius* and 48 individuals had the expected banding pattern of *G.*

breviceps. Structure was used to determine that there were two genetically distinct clusters of pocket gophers in Oklahoma (Figs. 3.5 and 3.6) that had a distribution pattern similar to that seen using RFLP data (Figs. 3.4). Excluding admixture individuals, 66 individuals were identified as *G. bursarius* and 48 individuals were identified as *G. breviceps* based on Structure clustering results. Both RFLP and Structure analyses showed agreement in terms of species identification (Appendix 3.1).

Boundary between G. bursarius and G. breviceps in Oklahoma

Heaney and Timm (1983) suggested that the boundary line between *G. bursarius* and *G. breviceps* meanders throughout central Oklahoma due to integration of several different soil types found in this region. Though species can be found in various soil types, *G. bursarius* prefers moderately sandy, well-drained soils, whereas *G. breviceps* prefers moist, riverine soils (Heaney and Timm 1983). The complex pattern of intertwining soils types found throughout Oklahoma may play a role in the shape of the species boundaries. Molecular analyses confirmed that the boundary line between *G. bursarius* and *G. breviceps* is occurring throughout central Oklahoma. Based on available *Cytb* RFLP and microsatellite results, the central boundary line runs through Logan, Payne, Oklahoma, and Cleveland Counties (Fig. 3.4, 3.6). Admixture individuals were found in Seminole County, so it is possible that the central boundary line also meanders through this county. The northern edge of the boundary line runs through Tulsa County. In addition to Tulsa County, the northern edge of the boundary line likely runs through Pawnee or Creek County. An admixture individual also was found in Marshall County, suggesting the southern boundary line runs through this county. The southern edge also likely runs through the south-central counties of McClain, Garvin, Murray, and Carter.

Additional samples are needed to confirm the exact position of the boundary in these regions. When comparing the RFLP (Fig. 3.4) and Structure (Fig. 3.6) results to the morphological data of Heaney and Timm (1983; Fig. 3.1), there was a similar pattern of the species boundary with regards to location and shape. There were, however, two samples that did not follow the expected pattern of species distribution.

Sample TK27149 collected in Hughes Co. and sample UCOK977 collected in McCurtain Co. were identified as *G. bursarius,* using both nuclear and mitochondrial markers (Appendix 3.1). As seen in Figures 3.4 and 3.6, these samples are located east of the proposed boundary line in central Oklahoma. To account for the possibility of contamination, both samples were re-extracted and reanalyzed separately from each other, and the results confirmed both individuals as *G. bursarius*. The fact that results from both nuclear and mitochondrial markers are agreeing with one another after re-testing means this was most likely not an error due to contamination or amplification of mitochondrial pseudogenes. There has been limited research done on pocket gopher dispersal mechanisms. Most studies that focus on dispersal are conducted in open fields and only span several acres (Miller 1964; Williams and Baker 1976; Williams and Cameron 1984; Daly and Patton 1990). Rivers serve as natural travel corridors for various animals (Puth and Wilson 2001). Habitat along rivers contains optimal soil composition for fossorial rodents (Connior and Risch 2009), including gophers. There have been numerous studies that have collected gophers residing along rivers (e.g., Patton and Yang 1977; Smith and Patton 1980). Additionally, pocket gophers have been reported to travel along rivers (Miller 1964; Cothran and Zimmerman 1985); however, there have been no studies focusing on dispersal along rivers in *Geomys*. There is a

possibility that *G. bursarius* could be expanding its range into eastern Oklahoma via rivers. Sample TK27149 was collected ~8 km north of the Canadian River and sample UCOK977 was collected ~4 km northeast of the Red River. The last study evaluating gophers from southeastern Oklahoma was conducted in the 1980s (Heaney and Timm 1983), meaning there is a possibility that *G. bursarius* has extended its range into eastern Oklahoma in the past 35 years. Additional molecular studies need to be conducted in southeastern Oklahoma to obtain a clearer understanding of possible dispersal mechanisms and the extent of dispersal by *G. bursarius* into this region.

Contact zones

Heaney and Timm (1983) identified multiple contact zones in central Oklahoma along the boundary between *G. bursarius* and *G. breviceps*. When examining the RFLP (Fig. 3.4) and Structure (Fig. 3.6) maps, there are multiple areas where the two species come into contact and potentially overlap with one another. Both species were collected in Cleveland, Logan, Oklahoma, Payne, and Tulsa Counties, suggesting there likely are contact zones in these areas. Definitive areas of overlap between the two species were identified in Cleveland and Tulsa Counties. The amount of overlap between species was determined using the measuring tool on ArcMap. This was done by measuring the distance of overlap between *G. bursarius* and *G. breviceps* found in the same location. The distance of the two species in Cleveland Co. was 5.25 km and the distance in Tulsa Co. was 12.01 km. Heaney and Timm (1983) hypothesized that the zone of contact between *G. bursarius* and *G. breviceps* was no wider than 8 km. Evaluation of contact zones found in this project indicate that they may be broader in some regions along the boundary line than previously suggested by Haynie and Timm (1983).

Hybridization

I identified four admixture individuals based on Structure results. When finding admixture individuals within a sample set, it is important to evaluate the possibility of incomplete linage sorting (ILS; also known as deep coalescence). ILS is a phenomenon that occurs when closely related species have not had enough time to evolve complete genetic divergence from one another. This means that two genetically distinct species can share certain alleles from their common ancestor. This phenomenon can make it difficult to distinguish between hybrid offspring and individuals that are sharing alleles with sister species due to ILS. For example, *Crotaphytus nebrius* (Sonoran collared lizard) and *C. collaris* (eastern collard lizard) clearly differ based on morphology, but carry mitochondrial haplotypes which are identical (McGuire et al. 2007). This most likely is due to ILS between the two recently diverged sister taxa. When comparing the possibility of ILS to hybridization, it is imperative to take note of the location in which each presumed hybrid individual was collected. Admixtures due to ILS should have no geographic pattern; admixtures due to hybridization should be restricted to the zone of contact.

Four individuals identified as admixtures were found in Logan, Seminole, and Marshall Counties (Fig. 3.6). No admixture individuals were found in Cleveland Co. where previous hybridization between *G. bursarius* and *G. breviceps* had been reported (Cothran and Zimmerman 1985). The most likely explanations are the rarity of hybridization and the number of samples obtained from each county. Two admixture samples, one in Logan County and one in Marshall County, were found along the proposed or suspected boundary line between the two species and likely represent hybrid

individuals. The presence of admixture individuals found farther from the boundary line (UCOK1004 and UCOK1005; Seminole County) can be explained in a few ways. Although the samples in Seminole County appear to be far from the boundary line shown in Figs. 3.4 and 3.6, examination of the boundary line described by Heaney and Timm (1983; Fig. 3.1) shows that it is not far from an area of overlap between the two species. The distance in which these samples were collected from the main boundary line could be explained by the meandering pattern of the boundary line due to soil integration. Additionally, as discussed previously, it also is possible that *G. bursarius* may be expanding eastward, thus pushing the contact zone between the two species further east. Hybrid zones have been reported to occur near rivers because they provide optimal soil type for burrowing (Cothran and Zimmerman 1985). Samples UCOK1004 and 1005 were collected ~14 km south of the North Canadian River. If *G. bursarius* is moving further east, using rivers as corridors, it would be feasible to observe hybrid individuals along rivers. Finally, there also is a possibility that these two individuals are F_2 offspring. Repeated backcrossing between hybrids and the parental species could explain why these individuals were found farther from the boundary line.

When comparing our molecular findings (Fig. 3.6) to Heaney and Timm's (1983) morphological findings (Fig. 3.1), the admixture samples found in this project are occurring close to areas where the two species have been reported or are expected to overlap with one another. Because these admixture individuals are found in or near areas of overlap suggests that they most likely are not occurring due to ILS but because they are hybrid individuals.

The RFLP results were based on mitochondrial DNA, which means the maternal species can be identified for each admixture individual. Sample UCOK918 was identified as *G. bursarius*, whereas samples OU4305, UCOK1004, and UCOK1005 were identified as *G. breviceps*. These results show that gene flow is occurring through both sexes and is bidirectional. Cothran and Zimmerman (1985) examined a contact zone in Norman, Cleveland County, Oklahoma and discovered one F_1 and multiple F_2/backcrossed individuals. Baker et al. (1989) found that admixture males were sterile and admixture females had reduced fitness between *G. knoxjonesi* and *G. bursarius*. Cothran and Zimmerman (1985) did not find sex-specific reduced fitness in Oklahoma hybrids, but instead found that these individuals had greatly reduced fitness compared to the parental species. Based on previous findings, it is possible that F_1 hybrids are reproducing and the individuals in this study could be F_1 or F_2 individuals. Further molecular analyses will need to be conducted to determine the possible extent of backcrossing in the Oklahoma hybrid zones.

Genetic diversity within and among Oklahoma gophers

Heterozygosity is a representation of genetic diversity within populations and can be a direct indicator of population fitness (Reed and Frankham 2000). The PIC values for all samples were relatively high (0.829) indicating that there are high levels of genetic diversity within each locus. However, the observed heterozygosity (mean HObs = 0.543) was lower than the expected heterozygosity (mean HExp = 0.849) across all loci and for each species (Table 3.3). Low heterozygosity values have been found in *Thomomys* (Nevo et al. 1974) and *Geomys* (Penney and Zimmerman 1976; Cothran and Zimmerman 1985), using allozymes. The levels of heterozygosity found in this study were within the

range expected for these microsatellite loci (Welborn et al. 2011) and were similar to those found in other studies of *Geomys* (Welborn and Light 2014; Chapter 2). Fossorial rodents have been reported to have low heterozygosity across a wide range of taxa (Nevo et al. 1974, 1990; Penney and Zimmerman 1976; Cothran and Zimmerman 1985). Nevo et al. (1990) suggested that low heterozygosity in fossorial rodents could be due to being constrained to a constant environment. This may explain the low levels of heterozygosity seen in *Geomys* in this and other studies.

Conclusions

There has been a limited amount of molecular research done on Oklahoma pocket gophers, which has led to a lack of understanding of species boundaries and contact zones throughout the state. Using both mitochondrial and nuclear markers, I was able to ascertain a clearer representation of the species boundary between *G. bursarius* and *G. breviceps* in central Oklahoma, confirming the proposed boundary based on morphological data. Four admixture individuals were identified, which indicates that there are most likely multiple hybrid zones along the boundary line where the two species overlap. Additionally, there is some evidence that the distribution of *G. bursarius* is shifting east in the state. Future research will focus on determining whether *G. bursarius* is expanding eastward, identifying the northern and southern boundaries of the contact zone, and in-depth evaluations of hybrid zones in the state. More focused sampling and genomic evaluation of hybrid zones in Oklahoma will be performed to thoroughly characterize the size and shape of the zones, as well as the level of introgression occurring between these two species.

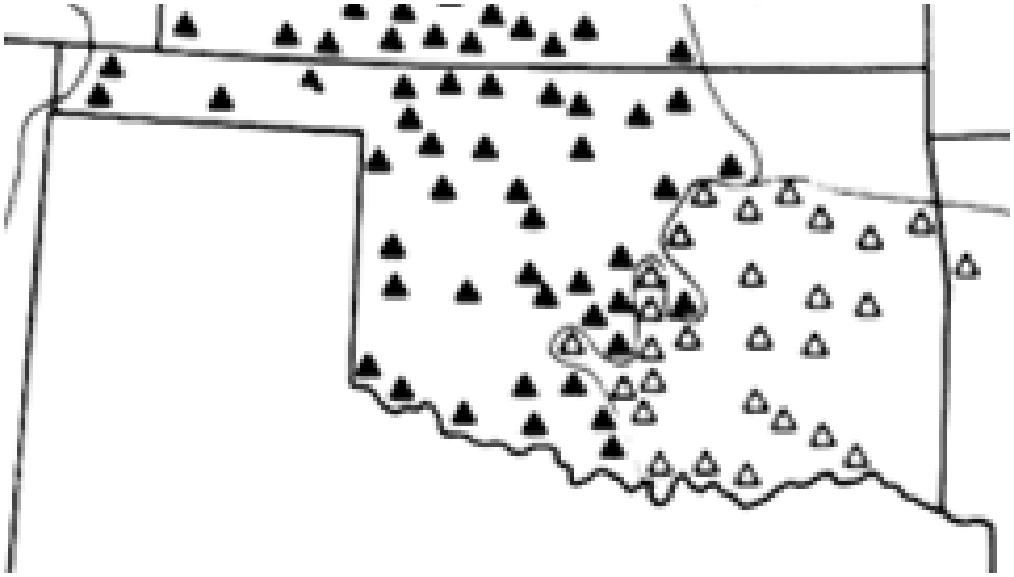

Figure 3.1: Map of Oklahoma showing *Geomys bursarius* (black triangles) and *G. breviceps* (white triangles) distributions in the state. Species identification was based on morphological data. The line through central Oklahoma represents the proposed boundary between the two species. Modified from Heaney and Timm (1983).

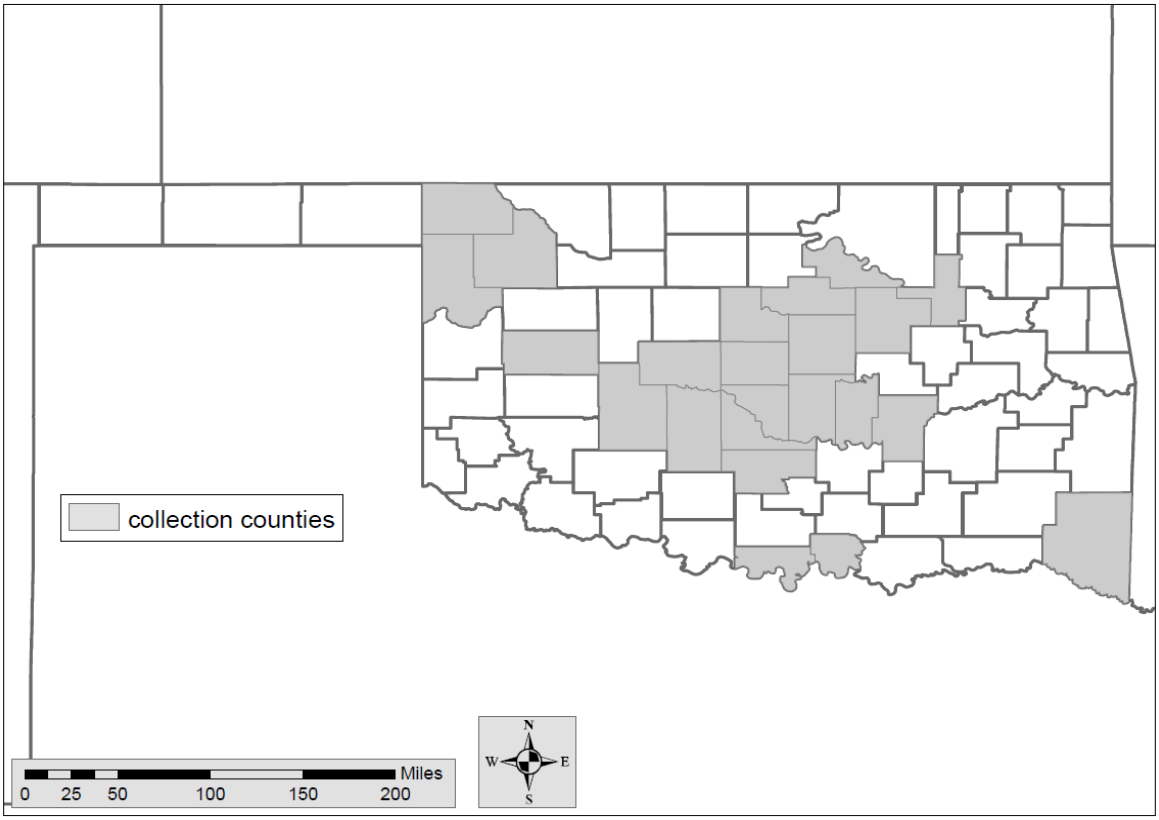

Figure 3.2: Map of Oklahoma showing collection localities of Oklahoma pocket gophers (shaded counties). The map was generated using ArcGIS (ESRI 2011).

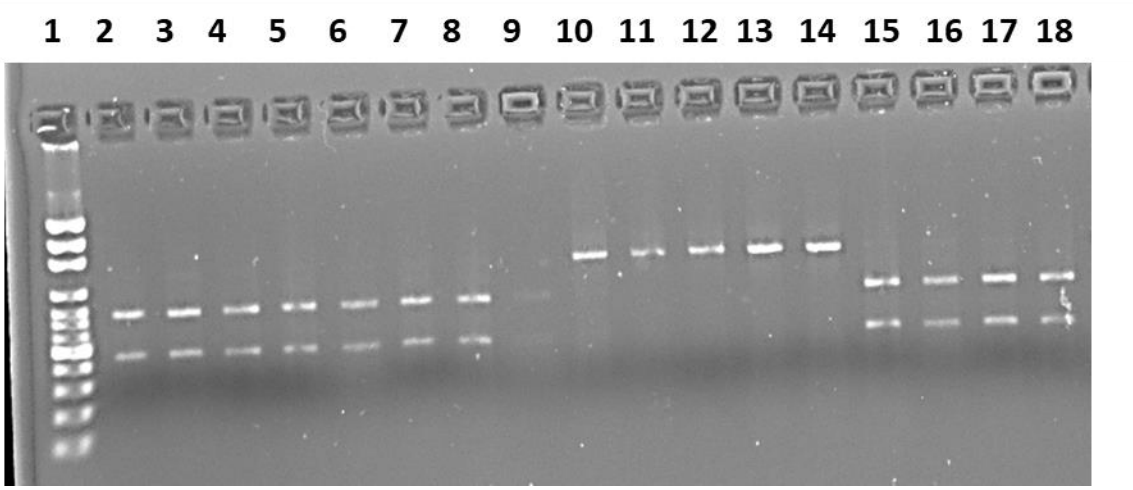

Figure 3.3: An example of the electrophoresis results for PCR-RFLP of the *Cytb* gene comparing *G. bursarius* to *G. breviceps* using the EcoRV enzyme. Lane one contains a 100 bp ladder. Samples were identified as *G. breviceps* if there were two fragments (413 bp and 727 bp; lanes 2-9, 15-18). Samples were identified as *G. bursarius* if the band was not cut (1140 bp; lanes 10-14).

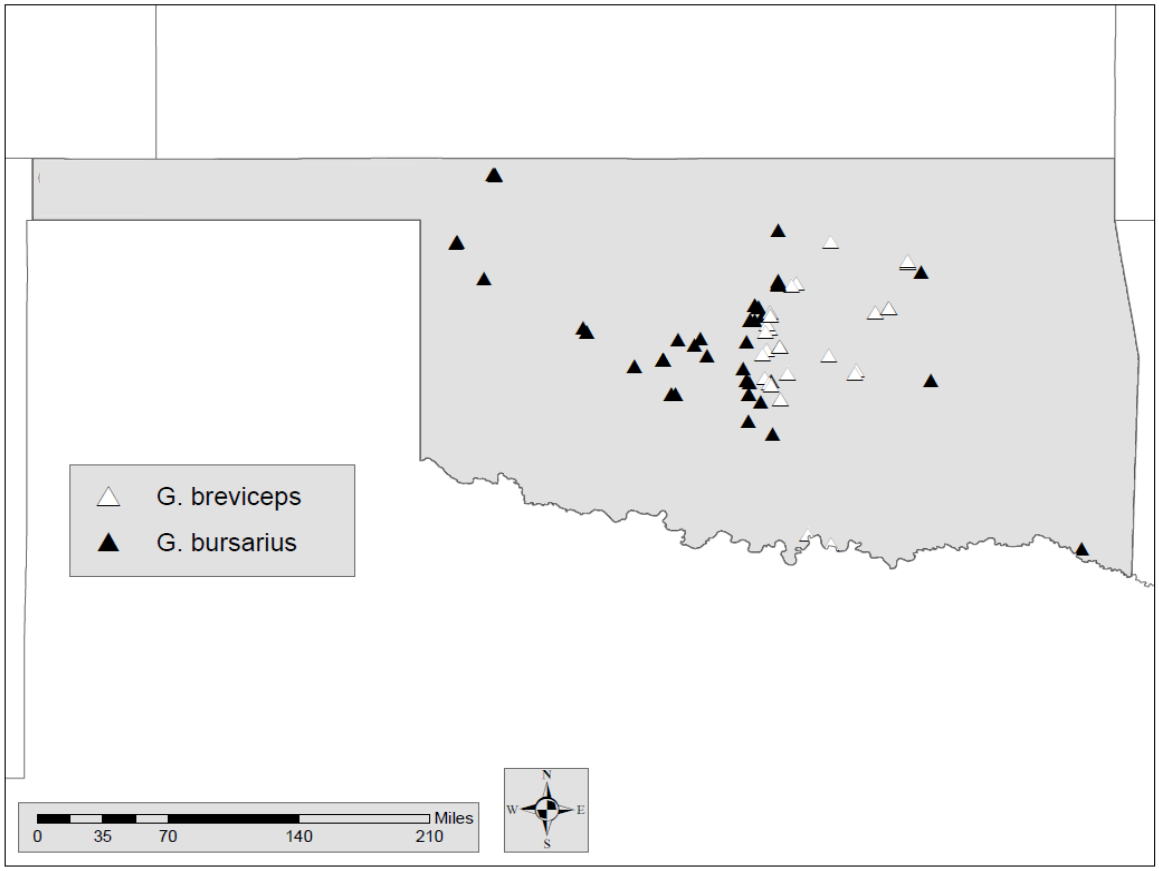

Figure 3.4: Map of PCR-RFLP results for the *Cytb* gene of Oklahoma pocket gopher identification. White triangles represent individuals identified as *G. breviceps* and black triangles represent individuals identified as *G. bursarius.* The map was generated using ArcGIS (ESRI 2011).

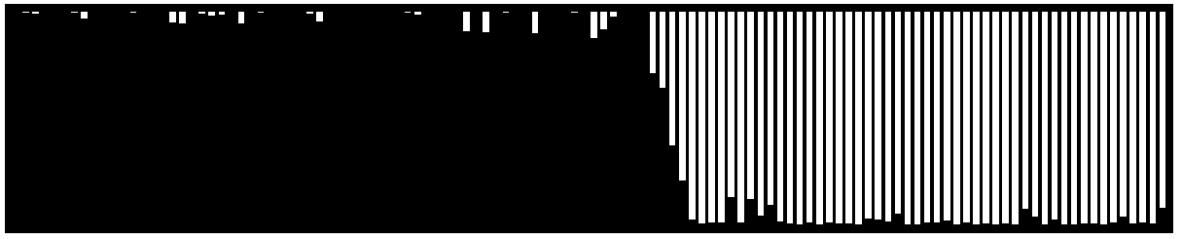

Figure 3.5: Barplot depicting clustering results from the Structure run consisting of all gopher samples collected in Oklahoma excluding the panhandle (*n* = 118). Each bar on the figure represents an individual. Colors correspond to the map in Fig. 3.6.

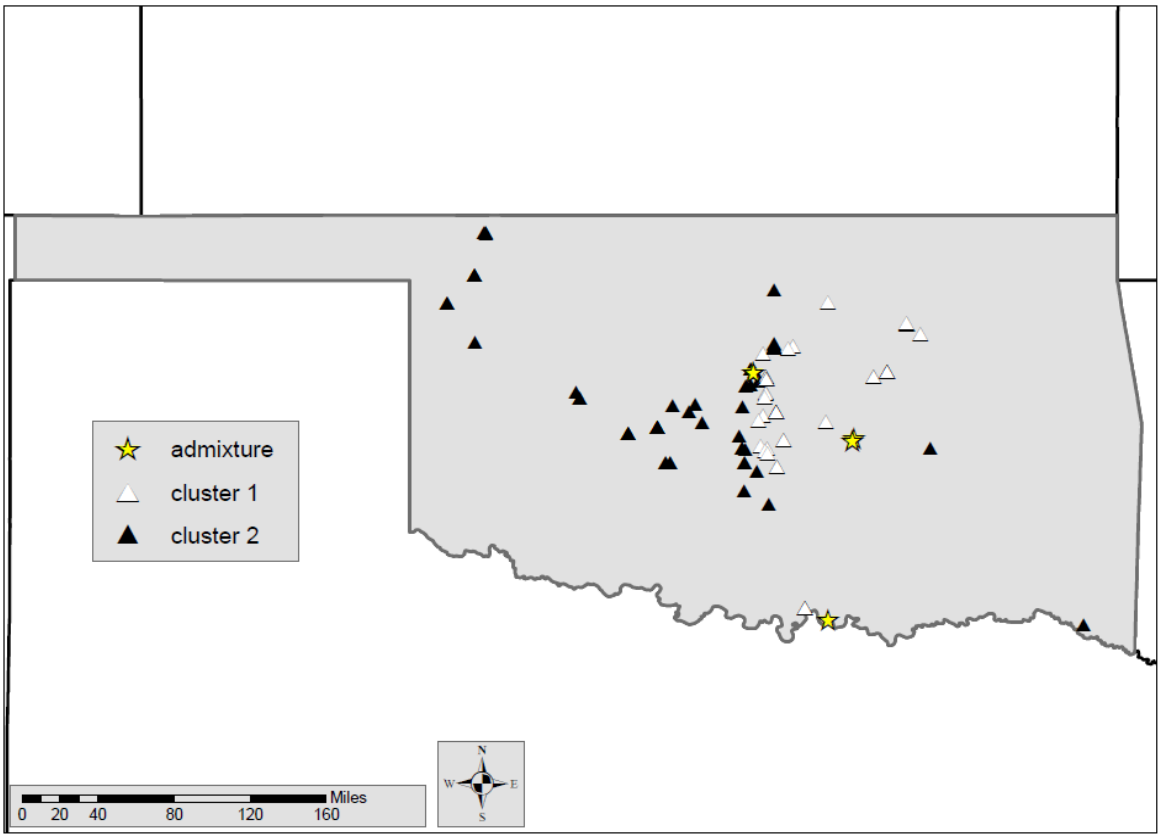

Figure 3.6: A map of cluster results from the Structure run consisting of all Oklahoma samples excluding the panhandle (n = 118). Black triangles correspond to the black lines on Fig. 3.5 and most likely represent *G. bursarius* individuals. The white triangles correspond to the white lines on Fig. 3.5 and most likely represent *G. breviceps* individuals. The stars represent admixture individuals. Admixture was determined based on assignment to a given cluster with a confidence level <80%. The map was generated using ArcGIS (ESRI 2011).

Table 3.1: Mitochondrial DNA cytochrome-*b* gene primers used for this study. The name, sequence, and source are provided for each primer.

Primer Name	**Primer Sequence**	**Source**
L14735	TGA AAA ACC ATC GTT GTT AAT TCA ACT	Elrod et al. 2000
H15906	CAT CTC CGG TTT ACA AGA CCT AAG TAA T	Elrod et al. 2000

Table 3.2: Microsatellite markers used in this study. The primer name, sequences (F = forward, R = reverse), size in base pairs (bp), annealing temperatures, and source are listed for each pair.

Primer Name	Primer Sequence	Size (bp)	Annealing Temperatures	Source
Gbr09	F- TGGCTCAAGTGAGAGCATCA	214	58°C, 56°C, 52°C	Welborn et al. 2011
	R- GGAGGAGGAACAAGCAATCA			
Gbr10	F- TAGTGCATGCTCTGGCTTTG	235	57°C, 55°C, 51°C	Welborn et al. 2011
	R- AAATGCCCTCCAGAAGGAAC			
Gbr15	F- CTCTCCCTCAGCTCAGCAGT	212	58°C, 56°C, 52°C	Welborn et al. 2011
	R- GTGTCCAGCCCAGTTATGCT			
Gbr25	F- CCTGGGAGACTAGCATGAGG	227	58°C, 56°C, 52°C	Welborn et al. 2011
	R- CACAAGAAAGCCAGAAGTGC			
Gbr27	F- TGATGACACGCTGACTTTCC	229	58°C, 56°C, 52°C	Welborn et al. 2011
	R- TGGAGGTGTAGCTCAAGTGG			
Gbr33	F- GTGGTAGTGGTGGTGTTTGC	227	56°C, 54°C, 50°C	Welborn et al. 2011
	R- ACACTGGAGTGTCTCATGTGG			
Tm1	F- TCACATACTAGCCCAAAGTCCTC	181	58°C, 56°C, 52°C	Steinberg 1999
	R- GTGGTAGAGCAAAAGAAGCTGAA			
Tm2	F- CCGGATCTTGGATTAGGCAT	173	58°C, 56°C, 52°C	Steinberg 1999
	R-GGCTGTTTTAATTTCCTTCATGT			
Tm7	F- TCTACTGAACCACCAGAAAATCAA	288	58°C, 56°C, 52°C	Steinberg 1999
	R- AGCACTGGACTTGAACACAAATAC			

Table 3.3: CERVUS (Marshall et al. 1998) results for *Geomys* samples collected throughout Oklahoma (*n* = 118). Separate analyses were run for samples identified as *G. breviceps* (*n* = 48) and those identified as *G. bursarius* (*n* = 66), excluding admixture individuals. Observed heterozygosity (HObs), expected heterozygosity (HExp), polymorphic information content (PIC), and number of alleles per locus are provided for all loci.

Locus	HObs	HExp	PIC	Alleles per locus
All Samples				
Gbr09	0.860	0.749	0.727	17
Gbr10	0.569	0.904	0.891	16
Gbr15	0.625	0.902	0.890	19
Gbr25	0.655	0.908	0.897	19
Gbr27	0.211	0.665	0.598	4
Gbr33	0.500	0.877	0.862	16
Tm1	0.313	0.920	0.910	22
Tm6	0.570	0.802	0.780	14
Tm7	0.588	0.914	0.904	20
Average	0.543	0.849	0.829	16.333
G. breviceps				
Gbr09	0.938	0.715	0.667	8
Gbr10	0.489	0.838	0.807	8
Gbr15	0.417	0.841	0.814	11
Gbr25	0.553	0.792	0.761	11
Gbr27	0.083	0.118	0.110	2
Gbr33	0.542	0.786	0.752	10
Tm1	0.271	0.879	0.857	14
Tm6	0.646	0.711	0.655	6
Tm7	0.479	0.851	0.825	13
Average	0.491	0.726	0.694	9.222
G. bursarius				
Gbr09	0.803	0.754	0.734	15
Gbr10	0.625	0.962	0.913	16
Gbr15	0.781	0.880	0.863	18
Gbr25	0.727	0.926	0.913	18
Gbr27	0.303	0.500	0.450	4
Gbr33	0.467	0.904	0.887	16
Tm1	0.344	0.911	0.897	20
Tm6	0.515	0.826	0.803	14
Tm7	0.667	0.919	0.906	20
Average	0.581	0.842	0.818	15.667

Appendix 3.1: Table representing all *Geomys* samples used in this study and their collection sites or museum sources. OU = Sam Noble Museum of Natural History; TTU = NSRL, The Museum of Texas Tech University; MSB = Museum of Southwestern Biology; UCOK = University of Central Oklahoma Natural History Museum (these samples were collected for this project). RFLP ID results and STRUCTURE clustering results are listed for each individual. Samples that were not able to be identified using PCR-RFLP were indicated with "N/A".

Sample ID	Source	State	County	Latitude	Longitude	RFLP ID	Structure ID
UCOK500	UCO	OK	Oklahoma	35.4787	-97.2170	*bursarius*	*bursarius*
UCOK518	UCO	OK	Oklahoma	35.4787	-97.2170	*bursarius*	*bursarius*
8/19/2013	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
8/22/2013	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
OU3202	OU	OK	Cleveland	35.2245	-97.3334	*breviceps*	*breviceps*
OU4305	OU	OK	Marshall	33.8863	-96.8204	*breviceps*	admixture
OU5295	OU	OK	McClain	35.0323	-97.3628	*bursarius*	*bursarius*
OU9423	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9424	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9425	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9426	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU9440	OU	OK	Woodward	35.2002	-97.2843	*bursarius*	*bursarius*
OU10088	OU	OK	Cleveland	35.1998	-97.2843	*breviceps*	*breviceps*
OU11726	OU	OK	Cleveland	35.1944	-97.4490	*bursarius*	*bursarius*
OU12041	OU	OK	Oklahoma	35.5238	-97.4725	*bursarius*	*bursarius*
TK27149	TTU	OK	Hughes	35.2097	-96.0454	*bursarius*	*bursarius*
TK182925	TTU	OK	Love	33.9714	-96.9969	*breviceps*	*breviceps*
TK182926	TTU	OK	Love	33.9714	-96.9969	*breviceps*	*breviceps*
TK182927	TTU	OK	Love	33.9714	-96.9969	*breviceps*	*breviceps*
TK182928	TTU	OK	Love	33.9714	-96.9969	*breviceps*	*breviceps*
TK182964	TTU	OK	Cleveland	35.0575	-97.2109	*breviceps*	*breviceps*
TK182965	TTU	OK	Cleveland	35.0575	-97.2109	*breviceps*	*breviceps*
MSB273626	MSB	OK	Caddo			N/A	*bursarius*
MSB273628	MSB	OK	Caddo			N/A	*bursarius*
MSB273629	MSB	OK	Caddo			N/A	*bursarius*
MSB273630	MSB	OK	Caddo			*bursarius*	*bursarius*
MSB273652	MSB	OK	Caddo			*bursarius*	*bursarius*

UCOK905	UCO	OK	Ellis	36.0283	-99.5065	*bursarius*	*bursarius*
UCOK910	UCO	OK	Oklahoma	35.6889	-97.4424	*bursarius*	*bursarius*
UCOK911	UCO	OK	Oklahoma	35.6887	-97.4462	*bursarius*	*bursarius*
UCOK912	UCO	OK	Oklahoma	35.6961	-97.4097	*bursarius*	*bursarius*
UCOK913	UCO	OK	Oklahoma	35.7533	-97.2935	*breviceps*	*breviceps*
UCOK914	UCO	OK	Oklahoma	35.7247	-97.3478	*bursarius*	*bursarius*
UCOK915	UCO	Ok	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
UCOK916	UCO	OK	Logan	35.8194	-97.4086	*bursarius*	*bursarius*
UCOK917	UCO	OK	Logan	35.8194	-97.4086	*bursarius*	*bursarius*
UCOK918	UCO	OK	Logan	35.7932	-97.3874	*bursarius*	admixture
UCOK919	UCO	OK	Logan	35.7980	-97.3783	*bursarius*	*bursarius*
UCOK920	UCO	OK	Cleveland	35.2116	-97.4741	*bursarius*	*bursarius*
UCOK921	UCO	OK	Cleveland	35.2116	-97.4741	*bursarius*	*bursarius*
UCOK922	UCO	OK	Canadian	35.5477	-97.8310	*bursarius*	*bursarius*
UCOK923	UCO	OK	Lincoln	35.6512	-97.8225	*breviceps*	*breviceps*
UCOK924	UCO	OK	Lincoln	35.6562	-97.8225	*breviceps*	*breviceps*
UCOK925	UCO	OK	Lincoln	35.6116	-97.8190	*breviceps*	*breviceps*
UCOK926	UCO	OK	Pottawatomie	35.4055	-96.8355	*breviceps*	*breviceps*
UCOK927	UCO	OK	Oklahoma	35.7533	-97.2935	N/A	*breviceps*
UCOK928	UCO	OK	Oklahoma	35.7533	-97.2935	*breviceps*	*breviceps*
UCOK929	UCO	OK	Payne	35.4238	-97.2294	*bursarius*	*bursarius*
UCOK930	UCO	OK	Payne	36.0042	-97.2290	*bursarius*	*bursarius*
UCOK931	UCO	OK	Payne	36.0101	-97.2294	*bursarius*	*bursarius*
UCOK932	UCO	OK	Payne	35.9897	-97.0868	*breviceps*	*breviceps*
UCOK933	UCO	OK	Logan	35.7316	-97.2889	*breviceps*	*breviceps*
UCOK934	UCO	OK	Logan	35.7316	-97.2889	N/A	*breviceps*
UCOK935	UCO	OK	Logan	35.9298	-97.3172	N/A	*breviceps*
UCOK936	UCO	OK	Payne	35.9733	-97.1241	*breviceps*	*breviceps*
UCOK937	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
UCOK938	UCO	OK	Logan	35.7316	-97.2906	*breviceps*	*breviceps*
UCOK939	UCO	OK	Logan	35.7316	-97.2906	*breviceps*	*breviceps*
UCOK940	UCO	OK	Cleveland	35.1847	-97.3004	*breviceps*	*breviceps*
UCOK941	UCO	OK	Cleveland	35.1698	-97.2831	*breviceps*	*breviceps*
UCOK942	UCO	OK	Oklahoma	35.4533	-97.3163	*breviceps*	*breviceps*
UCOK943	UCO	OK	Oklahoma	35.4207	-97.3496	*breviceps*	*breviceps*
UCOK944	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
UCOK945	UCO	OK	Canadian	35.5373	-98.0037	*bursarius*	*bursarius*
UCOK946	UCO	OK	Canadian	35.4933	-97.8768	*bursarius*	*bursarius*
UCOK947	UCO	OK	Canadian	35.4027	-97.7792	*bursarius*	*bursarius*
UCOK948	UCO	OK	Payne	35.9837	-97.2295	*bursarius*	*bursarius*
UCOK949	UCO	OK	Payne	35.9735	-97.2293	*bursarius*	*bursarius*
UCOK950	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*

UCOK951	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
UCOK952	UCO	OK	Oklahoma	35.4787	-97.2170	*breviceps*	*breviceps*
UCOK953	UCO	OK	Creek	35.7558	-96.4750	*breviceps*	*breviceps*
UCOK954	UCO	OK	Creek	35.7558	-96.4750	*breviceps*	*breviceps*
UCOK955	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK956	UCO	OK	Pawnee	36.3295	-96.8224	*breviceps*	*breviceps*
UCOK957	UCO	OK	Payne	35.9735	-97.2293	*bursarius*	*bursarius*
UCOK958	UCO	OK	Payne	35.9735	-97.2293	*bursarius*	*bursarius*
UCOK959	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK960	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK961	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK962	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK963	UCO	OK	Creek	35.7917	-97.3727	*breviceps*	*breviceps*
UCOK964	UCO	OK	Payne	35.9837	-97.2295	*bursarius*	*bursarius*
UCOK965	UCO	OK	Payne	35.9837	-97.2295	*bursarius*	*bursarius*
UCOK966	UCO	OK	Payne	35.9837	-97.2295	*bursarius*	*bursarius*
UCOK967	UCO	OK	Grady	35.0923	-98.0227	*bursarius*	*bursarius*
UCOK968	UCO	OK	Grady	35.0923	-98.0227	*bursarius*	*bursarius*
UCOK969	UCO	OK	Grady	35.0925	-98.0578	*bursarius*	*bursarius*
UCOK970	UCO	OK	Grady	35.0925	-98.0578	*bursarius*	*bursarius*
UCOK971	UCO	OK	McClain	34.8792	-97.4592	*bursarius*	*bursarius*
UCOK972	UCO	OK	Cleveland	35.3053	-97.4974	*bursarius*	*bursarius*
UCOK973	UCO	OK	Cleveland	35.2653	-97.1574	*breviceps*	*breviceps*
UCOK974	UCO	OK	Cleveland	35.2653	-97.1574	*breviceps*	*breviceps*
UCOK975	UCO	OK	McClain	35.0942	-97.4569	*bursarius*	*bursarius*
UCOK976	UCO	OK	McClain	35.0942	-97.4569	*bursarius*	*bursarius*
UCOK977	UCO	OK	McCurtain	33.8490	-94.8785	*bursarius*	*bursarius*
UCOK978	UCO	OK	Tulsa	36.0846	-96.1190	*bursarius*	*bursarius*
UCOK979	UCO	OK	Tulsa	36.1551	-96.2204	*breviceps*	*breviceps*
UCOK980	UCO	OK	Tulsa	36.1568	-96.2247	*breviceps*	*breviceps*
UCOK981	UCO	OK	Tulsa	36.1722	-96.2242	*breviceps*	*breviceps*
UCOK982	UCO	OK	Garvin	34.7753	-97.2714	*bursarius*	*bursarius*
UCOK983	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK984	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK985	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK986	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK987	UCO	OK	Caddo	35.3704	-98.1182	*bursarius*	*bursarius*
UCOK993	UCO	OK	Custer	35.5950	-98.7107	*bursarius*	*bursarius*
UCOK994	UCO	OK	Custer	35.5950	-98.7104	*bursarius*	*bursarius*
UCOK995	UCO	OK	Custer	35.6384	-98.7380	*bursarius*	*bursarius*
UCOK996	UCO	OK	Harper	36.8585	-99.4209	*bursarius*	*bursarius*
UCOK997	UCO	OK	Harper	36.8704	-99.4248	*bursarius*	*bursarius*

UCOK998	UCO	OK	Harper	36.8704	-99.4248	*bursarius*	*bursarius*
UCOK999	UCO	OK	Harper	36.8705	-99.4357	*bursarius*	*bursarius*
UCOK1000	UCO	OK	Ellis	36.3227	-99.7155	*bursarius*	*bursarius*
UCOK1001	UCO	OK	Ellis	36.3215	-99.7170	*bursarius*	*bursarius*
UCOK1002	UCO	OK	Ellis	36.3193	-99.7221	*bursarius*	*bursarius*
UCOK1004	UCO	OK	Seminole	35.2900	-96.6237	*breviceps*	admixture
UCOK1005	UCO	OK	Seminole	35.2650	-96.6358	*breviceps*	admixture

CHAPTER 4

GENERAL SUMMARY AND FUTURE RESEARCH

The purpose behind biological research is to gain a better understanding of the living world. Research that focuses on clarifying the identification and taxonomy of a species is imperative because species are the fundamental unit for understanding ecology, biodiversity, conservation efforts, and other biological issues (Bradley and Baker 2001). My research focused on examining Oklahoma *Geomys* species identification, species distribution, contact zones, and hybridization.

Using molecular markers, I was able to identify three genetically distinct species of *Geomys* in Oklahoma based on assessment of 146 individuals. I concluded that *G. breviceps* is found mainly in the eastern half of Oklahoma, *G. bursarius* is restricted mostly to the western half of Oklahoma, and *G. jugossicularis* is found in the Oklahoma panhandle (Cimarron and Beaver Counties) and Texas panhandle (Dallam and Hartley Counties). This is the first time *G. jugossicularis* has been confirmed to occur in Oklahoma and Texas. Additionally, there is some evidence that *G. bursarius* is expanding its range into eastern Oklahoma. It also is found in Beaver County in the Oklahoma panhandle.

The boundary lines between the three *Geomys* species are now more clearly defined in Oklahoma. The boundary between *G. bursarius* and *G. breviceps* is located in central Oklahoma, running through Logan, Payne, Tulsa, Oklahoma, and Cleveland Counties. Based on the presence of admixture individuals, the boundary line also may run through Seminole and Marshall Counties. Molecular data supported the suggested

boundary by Heaney and Timm (1983), which was based on morphological data. The southern boundary line between *G. bursarius* and *G. jugossicularis* most likely occurs in the Oklahoma and Texas panhandles.

My research has shown that there are extensive contact zones between the species in central Oklahoma and in the panhandle. I was able to evaluate contact zone width by measuring the distance between overlapping individuals. There were two counties in which contact zone width could be measured. The overlap of the two species in Cleveland Co. was 5.25 km and the overlap in Tulsa Co. was 12.01 km. Heaney and Timm (1983) hypothesized that the zone of contact between *G. bursarius* and *G. breviceps* was no wider than 8 km. The results of this research suggest that the contact zones likely are wider in some regions along the boundary line than suggested by Heaney and Timm (1983).

Within these contact zones there is the potential for hybridization. A total of eight admixture individuals were identified in this project. Four admixture individuals were found between *G. bursarius* and *G. breviceps* in Seminole, Logan, and Marshall Counties, which means there are likely multiple hybrid zones between these two species in the central region of the state. Four admixture individuals were found between *G. jugossicularis* and *G. bursarius* in the Oklahoma panhandle in Beaver and Cimarron Counties. Two admixture individuals found in Beaver County were roughly 23 km apart, which indicates that there is a possibility of multiple hybrid zones located throughout Beaver County.

Though hybrid zones were not studied in detail for this project, I was able to hypothesize the basic structure of hybrid zones between *G. bursarius* and *G. breviceps* in

central Oklahoma based on preliminary findings, as well as previous research. Hybridization in central Oklahoma was hypothesized in the 1950s (Baker and Glass 1951), and confirmed in the 1980s (Bohlin and Zimmerman 1982) and with this project in 2018. With this information, I can conclude that hybrid zones have been in the same relative locations in Oklahoma for at least the past 35 years and perhaps as long as 67 years, which means these hybrid zones may be stable in this region at least for short time periods. Cothran and Zimmerman (1985) suggested that the hybrid zone in Norman, Oklahoma was dynamic, meaning the hybrid zone may move throughout time. This is because there are no ecological barriers separating the two species in central Oklahoma and the hybrid zones are not confined to a specific location. The lack of ecological barriers and possible fluidity of the hybrid zones, and the short term stability of these zones over time is best explained by the tension zone model of hybridization.

As discussed in Chapter 1, the tension zone model describes the maintenance of these hybrid zones as a balance between dispersal and selection against hybrid offspring (Gay et al. 2008). The difference between the tension zone model and the dynamic equilibrium model is that ecological factors do not play a role in the maintenance of hybrid zones under the tension zone model (Nichols 1989; Gay et al. 2008), therefore zones are not restricted to a specific habitat. There are no known ecological barriers for pocket gophers in central Oklahoma that restrict the size and shape of the hybrid zones. Additionally, hybrid offspring have reduced fitness compared to the parental species within tension zones (Hewitt 1988; Nichols 1989; Gay et al. 2008). There have been numerous studies that have reported reduced fitness in hybrid offspring of pocket gophers when compared to the parental species (Pembleton and Baker 1978; Tucker and

Schmidly 1981; Baker et al. 1989). Cothran and Zimmerman (1985) reported that the hybrid offspring of *G. bursarius* and *G. breviceps* in central Oklahoma would most likely produce unbalanced gametes and therefore have reduced fitness. Tension zones have been reported to occur in areas with low population densities (Nichols 1989). Because of soil integration across Oklahoma, as well as surrounding states where *Geomys* occur, the populations are described as being distributed in a "mosaic pattern" (Penney and Zimmerman 1976; Cothran and Zimmerman 1985). These populations often have low densities due to their secluded and discontinuous nature. It is for these reasons that the tension zone model is the most likely option to describe the hybrid zones between *G. bursarius* and *G. breviceps* found in central Oklahoma.

The overall goals of this research were to gain a better understanding of species boundaries, contact zones, and hybridization in Oklahoma *Geomys* pocket gophers. My research has provided an opportunity to advance the growing knowledge of cryptic species and the occurrence of hybridization between these closely related species. Most importantly, my project has aided in a broader understanding of *Geomys* species in Oklahoma which will allow for future research opportunities. This study represents the first step of a large collaborative project aimed at comparing hybridization rates and speciation mechanisms across multiple hybrid zones.

Future research will focus on collecting samples from south-central Oklahoma, southeastern Oklahoma, and the Oklahoma panhandle in order to fully represent Oklahoma *Geomys* diversity. Samples also will be collected from the Texas panhandle to determine the range of *G. jugossicularis* in this region. A better representation of the

pocket gophers in Oklahoma as well as the Texas panhandle will greatly aid in the growing knowledge of *Geomys'* distribution.

Future research also will focus on a more detailed evaluation of the hybrid zones found within central Oklahoma and the panhandle. Samples will be collected from the regions in which the admixture individuals were found, as well as from the previously identified hybrid zone located in Norman, Oklahoma (Bohlin and Zimmerman 1982; Cothran and Zimmerman 1985). Individuals will be characterized using a whole-genome approach. Additionally, multiple hybrid zones between species with varying levels of genetic divergence will be compared. Evaluation of hybrid zones will include the spatial parameters of each zone (e.g., size and shape), frequency of hybrids within the zone, types and frequencies of hybrid genotypes (e.g., F_1, F_2, etc.), directionality of introgression, genetic structure within the zone (e.g., distribution of genotypes), and the model of maintenance (e.g., hybrid superiority, hybrid equilibrium, tension zone). Hybrid zone characteristics will be evaluated and compared between different zones and different species pairs to determine if similar characteristics are occurring and to determine if the same mechanisms are acting in each zone. By determining common characteristics between zones, there will be a better understanding of the mechanisms necessary for speciation. With use of the whole genome approach, future research will be able to determine which regions of the genome are under selection in each hybrid zone. It is expected that some of the regions under selection will differ between the zones as each region is experiencing different ecological variables (e.g., soil type, climate). Consequently, identification of shared regions under selection will indicate genes important in the speciation process for this group. It might therefore be possible to

identify “speciation genes” for *Geomys*. Not only do hybrid zones need to be evaluated in depth, but populations around these hybrid zones should be evaluated using molecular techniques to observe possible selective pressures of individuals located just outside the hybrid zone and determine what evolutionary mechanisms are playing a role in keeping these species genetically distinct from one another.

In depth evaluation of hybrid zones between genetically distinct taxa are important to studies of evolutionary biology and systematics (Barton and Hewitt 1985; Slatkin 1987). These studies allow insight into processes associated with pre-mating and post-mating isolating mechanisms, speciation, origin of novel genetic material, introgression, heterozygote fitness, and many other aspects of genetic and reproductive isolation (Barton and Hewitt 1985; Hewitt 1988; Baker et al. 1989; Harrison 1990; Abbott et al. 2013). Ultimately, this research will extend to hybrid zones in other taxonomic groups (especially lesser-known vertebrates), as a new model for evaluating hybridization in an evolutionary context.

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