GENETIC ANALYSIS OF BOBCATS (*LYNX RUFUS*) AND OCELOTS (*LEOPARDUS PARDALIS*) IN A FRAGMENTED LANDSCAPE USING NONINVASIVE SAMPLING

A Thesis

by

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ABSTRACT

Genetic Analysis of Bobcats (*Lynx rufus*) and Ocelots (*Leopardus pardalis*) in a Fragmented Landscape Using Noninvasive Sampling

(December 2017)

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Bobcats (*Lynx rufus*) are highly mobile, occupy diverse habitat types, and are well-suited for adaptation in response to anthropogenic changes on the landscape. These abilities make bobcats valuable indicators of landscape connectivity, which is essential for the viability of wildlife populations. Understanding patterns of gene flow among bobcat populations can help evaluate habitat corridors through urban environments, such as the Lower Rio Grande Valley of South Texas. I used scat sampling and landscape-genetic methods to determine the impact of habitat fragmentation on genetic connectivity of bobcats in South Texas. The noninvasive scat sampling strategy was an efficient method for collecting genetic samples of bobcats, but appears unsuccessful for monitoring ocelot *(Leopardus pardalis)* populations. I found evidence for restricted dispersal of bobcats in fragmented habitat patches through analysis of spatial autocorrelation and contemporary gene flow. Bobcat populations in contiguous rangeland habitats appeared panmictic, while fine-scale spatial autocorrelation in fragmented areas indicated restricted dispersal. Estimates of migration rates and direction of gene flow indicated that bobcats within the fragmented areas are likely to remain or disperse to rangeland if possible, but dispersal rarely occurred into the fragmented areas from the rangeland. Monitoring connectivity of habitat corridors will be important for the long-term conservation of wildlife in the region, including the two remaining U.S. ocelot populations.

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CHAPTER I

LITERATURE AND METHODS REVIEW

BOBCAT ECOLOGY

Taxonomy

The bobcat (*Lynx rufus*) is one of 38 living species in the cat family (Felidae) (Wozencraft 1993, Hansen 2006). The genus *Lynx* is one of eight major lineages within Felidae, which includes the bobcat, Canada lynx (*Lynx canadensis*), Eurasian lynx (*L. lynx*), and Iberian (Spanish) lynx (*L. pardinus*)(Wozencraft 1993, Johnson and O'Brien 1997). Members of the genus *Lynx* share a common ancestor, the Issoire lynx (*L. issiodorensis*), which originated in South Africa 10-15 million years ago (mya)(Werdelin 1981, Johnson and O'Brien 1997). From South Africa, the Issoire lynx expanded its range northeast into North America 2-5 mya, evolved into an intermediate form (*L. issiodorensis kurteni*), and then into the bobcat (Werdelin 1981, Hansen 2006). The oldest bobcat fossil was found in Texas and dates to 2.4-2.5 mya (Hansen 2006). Twelve subspecies of bobcat are currently recognized (Hall 1981). However, there are few discrete physical characteristics for distinguishing subspecies, which makes individual assignment difficult without the use of genetic data (McCord and Cardoza 1982, Reding et al. 2012).

Distribution

Bobcats are the most common native felid in North America, with an estimated 2-3 million individuals (Roberts and Crimmins 2010). The bobcat has the widest distribution of any native North American felid, ranging from the East to the West Coasts and southern Canada through central Mexico (Anderson and Lovallo 2003). Fossil records suggest that bobcats were historically abundant and widespread, and they have maintained most of their historic range

Chapter I of this thesis is written in the style of *The Journal of Wildlife Management.*

despite extirpation from parts of the Midwest and mid-Atlantic (Woolf and Hubert 1998, Hansen 2006, Graham and Lundelius 2010).

Mating

Bobcat mating behavior is polygynous (Anderson and Lovallo 2003). Female bobcats are sexually mature at about 9-12 months of age, but yearling pregnancies are rare; males become sexually mature in their second year (Hansen 2006). However, age of sexual maturity may fluctuate with bobcat density and prey availability (Rolley 1985). In most of the bobcat's range, breeding peaks from February to April, but may occur year-round in southern latitudes (Blankenship and Swank 1979). Gestation ranges from 50-70 days (Lariviére and Walton 1997, Sunquist and Sunquist 2002). Female bobcats can reproduce for 6-8 years, with up to 6 kittens per litter (McCord and Cardoza 1982, Sunquist and Sunquist 2002, Reid 2006); average litter size in South Texas is 3 kittens (Blankenship and Swank 1979). Females are solitary, raise litters without assistance from the male, and typically only interact with males during breeding (Bailey 1974, Sunquist 1987, Hansen 2006). The maximum life span for a bobcat in the wild is about 11 years (Crowe 1975).

Dispersal

Bobcat kittens are weaned at 2 months, travel with their mother until approximately 9 months of age, and disperse before the next litter is born (Hansen 2006, Reid 2006). Natal dispersal is essential for bobcats to attain resources, mating, and to avoid inbreeding (Ferreras et al. 2004). Juveniles have a high mortality rate when transitioning to a solitary lifestyle (Hansen 2006). Bobcats commonly disperse 20-40 km, but dispersal distances are highly variable and some individuals have traveled >150 km (Hansen 2006, Knick and Bailey 1986). Dispersal is sex-biased in that male bobcats travel furthest while females remain close to their natal home range, typical for mammalian behavior (Janecka et al. 2007, Croteau et al. 2010).

Habitat Use

Bobcats occupy habitats ranging from coastal wetlands to mountains, and dense forests to desert thornshrub (Hansen 2006). Bobcats can even occur in suburban environments (Riley et al. 2006). At finer scales, prey availability and understory cover for hunting, resting, and denning influence bobcat distribution (McCord and Cardoza 1982, Lariviére and Walton 1997, Hansen 2006). High-quality habitat for bobcats includes woody vegetation that provides horizontal cover dense enough to conceal a stalking bobcat, but transparent enough for the bobcat to observe prey (Hansen 2006). Bobcats prefer riparian areas to grassland habitat, particularly during droughts (Lawhead 1984). Bobcats may shift habitat use in response to fluctuations in prey availability and environmental conditions (Larivier and Walton 1997, Blankenship 2000, Janecka 2002). Females may require and select for higher quality habitat compared to males to increase the chances of success in rearing kittens (Bailey 1981, Anderson and Lovallo 2003). Den sites are areas of thermal cover and shelter from rain, which include under rocky outcrops, felled trees, brush piles, and human-made structures.

Home Range

Home range of a bobcat is defined as the area a solitary bobcat uses during normal activities, such as hunting, mating, and caring for the young (Burt 1943). Average bobcat home-range size is 30 km^2 but varies by diet, sex, and geographic region (Gittleman and Harvey 1982). Average home-range size on the Welder Wildlife Refuge in South Texas was approximately 7 km^2, but ranged from 1-30 km^2 (Janecka et al. 2006). Females have smaller home ranges that rarely overlap those of other females (Bailey 1974). Male home ranges are larger and can overlap multiple female home ranges, as well as those of other males (Lariviére and Walton 1997, Sunquist and Sunquist 2002). Transient bobcats, those dispersing or without established home ranges, may pass through established territory of resident bobcats without aggressive interaction (Bailey 1974). Home ranges shift or are colonized by a transient when a resident bobcat is

removed (McCord and Cardoza 1982, Lariviére and Walton 1997). Bobcats sometimes establish residence in one location over its lifetime (Bailey 1974, Litvaitis et al. 1987), but can change home range size in response to prey availability (Blankenship 2000).

Diet and Scatology

Male bobcats weigh an average of 10 kg, ranging from 5-17 kg (Reid 2006), and weigh 25% to 75% more than females (Sunquist and Sunquist 2002, Anderson and Lovallo 2003). Bobcats are strictly carnivorous hunters. Their diet mainly consists of rabbits, rodents, and small mammals, with occasional deer, birds, reptiles, and fish (Lariviére and Walton 1997). Bobcats are capable of killing adult white-tailed deer (*Odocoileus virginianus*) and mule deer (*O. hemionus*), but often prefer easier and less risky hunting when lagomorphs and rodents are abundant (Beasom and Moore 1977). Prey items vary across the range of the bobcat and can fluctuate according to season or availability (Beasom and Moore 1977, Blankenship 2000). The main prey items for bobcats in South Texas are hispid cotton rats (*Sigmodon hispidus*), Southern Plains wood rats (*Neotoma micropus*), and eastern cottontails (*Sylvilagus floridanus*; Blankenship 2000).

Felids require high-protein diets and thus have digestive enzymes capable of metabolizing large quantities of meat (Golley et al. 1965). Since meat is easier to digest than plant matter, felids have short and efficient digestive tracts compared to other carnivores that supplement their diet with vegetation (Hansen 2006). This lighter gut supports quick bursts of movement used by felids in ambushing prey (Houston 1988, Hansen 2006). Bobcats are able to digest > 90% of their food, and are better than canids at digesting the small bones of their prey (Johnson and Aldred 1982). Although seeds are seldom found in bobcat scat, grasses are sometimes consumed to help remove parasites from their intestine (Elbroch 2003). Bobcat scats are often dense, not twisted, and segmented with blunt ends or tapered at one end (Elbroch 2003). However, this description tends to be more accurate in arid environments, whereas bobcat

scats in humid environments have greater variation in appearance and are often confused with coyote (*Canis latrans*) scats when diets overlap (Murie and Elbroch 2005).

Urine and scat are major sources of communication for bobcats. Bobcats defecate, urinate, or spray from their anal glands along trails to mark territory or find available mates (Bailey 1974, Hansen 2006). Scent marking occurs at trail junctions or at the base of a tree, and has been observed along a highly used trail as much as 30 times in less than 0.5 km (Elbroch 2003). Scat latrines are commonly associated with strategic locations, such as trail junctions, bridges, or large fallen trees (Elbroch 2003; T. L. Blankenship, Welder Wildlife Foundation, personal communication). Bobcats will scrape the substrate around their scat or urine, and may deliberately bury their scat (Elbroch 2003).

Interspecific Competition

The most significant competitor to the bobcat is the coyote due to overlapping geographic distribution, habitat type, and prey items (Hansen 2006, Reid 2006). Coyotes are better adapted to anthropogenic disturbances than bobcats in part due to their omnivorous diet and wider range of habitat selection (Buskirk et al. 2000, Hansen 2006). Direct competition has been observed from coyotes preying on bobcats (Anderson 1987, Litvaitis and Harrison 1989, Fedriani et al. 2000). In Texas, abundance of bobcats and prey increased in a study that culled coyote populations, suggesting indirect competition (Henke and Bryant 1999). Domestic dogs (*Canis lupus familiaris*) represent antagonists for bobcats in suburban areas, as large dogs kill bobcats and bobcats kill small dogs (Lembeck 1978; M. E. Tewes, Texas A&M University-Kingsville, personal communication).

OCELOT ECOLOGY

Taxonomy

The ocelot (*Leopardus pardalis*) is one of 7 living species in the genus *Leopardus* of the cat family (Felidae)(Wozencraft 1993, Hansen 2006). The genus *Leopardus,* also known as the "ocelot lineage," is one of 8 major lineages within Felidae, which includes the ocelot, Andean mountain cat (*L. jacobita*), Geoffroy's cat (*L. geoffroyi*), kodkod (*L. guigna*), margay (*L. wiedii*), pampas cat (*L. colocolo*), and oncilla (*L. tigrinus*)(Wozencraft 1993, Sunquist and Sunquist 2002). The ocelot lineage was diverged within Felidae about 8 mya (O'Brien and Johnson 2007). The most distinguishing feature of the ocelot lineage is its 18 pairs of chromosomes, while the other cats have 19 pairs of chromosomes (Sanderson and Watson 2011). The 7 species in the ocelot lineage are found in Central or South America. There are 10 recognized subspecies of ocelots, with the ranges of *L. p. albescens* and *L. p. sonoriensis* extending into South Texas and Arizona, respectively (Wozencraft 1993).

Distribution

Ocelots range from the southern tip of Texas and western Mexico through Central and South America into northern Argentina. Until the 1800s, their historical range extended through eastern Texas into parts of Louisiana and Arkansas, as well as in parts of Arizona (Leslie 2016). Ocelots were first listed as endangered in the U.S. in 1982 (U.S. Department of Interior, Fish and Wildlife Service 1982), and currently, there are < 80 ocelots in the U.S. (Tewes 2017). The main cause of population decline for ocelots was the loss of habitat and over-exploitation (Laack 1991). Within the U.S., the subspecies *L. p. albescens* occurs in 2 breeding population in South Texas, on the Laguna Atascosa National Wildlife Refuge, and on private ranches in Cameron, Willacy, and Kenedy counties (Tewes and Everett 1986, Janecka et al. 2006). The 2 populations are < 30 km apart yet seem to be genetically isolated (Janecka et al. 2011). The ocelots are not uniformly distributed across the landscape, as > 95% of the native thornshrub and riparian forests

that they inhabit have been removed from the Lower Rio Grande Valley of South Texas for agriculture and urban development (Purdy 1983, Laack 1991). The subspecies *L. p. sonoriensis* is occasionally detected in Arizona (Holbrook et al. 2011), but there is no evidence for a breeding population of this subspecies in the U.S. (Leslie 2016).

Mating

Ocelot mating behavior is polygynous. Female ocelots are sexually mature at about 18-23 months of age (Navarro-Lopez 1985, Tewes 1986), while a male's sperm may not be viable until approximately 30 months of age (Mondolfi 1986). Female ocelots are polyestrous. Estrus can occur every 6 weeks in captivity but typically occurs every 4-6 months in the wild and lasts approximately 7-10 days (Eaton 1977, Murray and Gardner 1997). After a 2.5-month gestation, a female ocelot may birth litters of 1-2 kittens and can produce a litter every 2 years (Emmons 1988). Litters may be born at any time of the year, with peaks in Texas between August and December (Tewes 1986, Laack 1991). Ocelot dens are located in hollow trees, caves, and dense vegetation for concealment (Davis 1974). A female ocelot uses multiple denning locations for a single litter, which typically consists of 2-4 dens spaced 100-300 m apart (Laack 1991; Laack et al. 2005). The maternal ocelot raises the litter without assistance from the paternal male (Murray and Gardner 1997).

Dispersal

Ocelot kittens travel with their mother until they disperse from their natal range, between 14 and 35 months of age (Laack 1991). Juvenile ocelots in South Texas may make exploratory movements for 2-8 months before settling into a new home range up to 10 km from their natal range (Navarro-Lopez 1985, Laack 1991). Ocelot mortality rates are high during dispersal from the natal range (Ludlow and Sunquist 1987). Adult ocelots are solitary, but brief intersexual interactions may occur outside of breeding periods (Murray and Gardner 1997).

Habitat Use

Ocelots are habitat specialists, and occupy habitat that provides dense cover across their range (Ludlow and Sunquist 1987, Tewes and Schmidly 1987). Suitable habitat includes tropical dry forests, tropical humid forests, riparian forests, and dry thornshrub where populations are abundant in Central and South America (Tewes and Schmidly 1987, Horne 1998). However, ocelots in Texas are restricted to patches of habitat that contain dense thornshrub (Shindle and Tewes 1998, Jackson et al. 2005), with almost completely closed canopy (Navarro-Lopez 1985, Tewes 1986, Laack 1991). Ocelots are primarily nocturnal or crepuscular, with less activity during the day than bobcats (Leonard 2017). Ocelots will occasionally move short distances through open habitats (Emmons et al. 1989, Sunquist and Sunquist 1989, Laack 1991), although they show a stronger aversion to open areas compared to bobcats (Horne et al. 2009). Dispersing ocelots will use less favorable habitat corridors, having been observed using narrow corridors of brush between 5-100 m wide along rivers and drainages (Laack 1991).

Home Range

Ocelot home-range size in South Texas ranges from 2-17 km^2, averaging 6 km^2 for males and 3 km^2 for females (Navarro-Lopez 1985, Tewes 1986, Laack 1991). Female home ranges rarely overlap those of other females, but male home ranges are larger and can overlap 1-3 female home ranges, as well as those of other males (Tewes 1986, Laack 1991). Resident ocelots with established home ranges will tolerate transient ocelots passing through their territory (Laack 1991), although direct territorial defense has been observed (Emmons 1988, Horne 1998). Ocelots show high fidelity towards their home range; individuals occupied the same areas over the course of a 4-yr study in South Texas (Laack 1991). Younger ocelots may displace older individuals from their home ranges (Emmons 1988). Adult ocelots in Venezuela visited the entire boundary of their home range every few days (Emmons 1988).

Diet and Scatology

Ocelots have similar diets to bobcats. They are strictly carnivorous hunters, with a diet of small mammals, reptiles, birds, and fish (Emmons 1988), varying by season or availability (Ludlow and Sunquist 1987). Over 90% of their mammalian prey weighs < 1 kg (Ludlow and Sunquist 1987, Murray and Gardner 1997). Adult ocelots weigh between 7-14 kg (Tewes 1986, Sanderson and Watson 2011), averaging 9.5 kg, and consume 550-850 g of meat/day (Emmons 1987).

Ocelots likely defecate daily as suggested by their consumption rate of small prey (Emmons 1987). Ocelot scats are usually segmented, 1.3-2.2 cm diameter (Elbroch 2003), and appear similar to bobcat scats, especially when diets overlap (Murie and Elbroch 2005; D. R. Taylor, personal observation). Ocelots mark their territory and communicate with urine, similar to bobcat scent marking (Horne 1998). Ocelots defecate and spray urine at latrine sites that probably play a role in interspecific and intraspecific communication (King et al. 2017). Latrine sites may be used by multiple individuals and are typically placed at the base of a tree or other large object (Murray and Gardner 1997). Ocelot scats have been collected along roads, trails, along sandy edges of riparian zones, and near resting sites in Central and South America (Ludlow 1986, Murray and Gardner 1997, Wultsch et al. 2014). However, scat-marking behavior of ocelots in Texas has not been described.

Interspecific Competition

The southern range of the bobcat and northern range of the ocelot overlap in South Texas and Mexico (Tewes and Schmidly 1987). Bobcats are less of a habitat specialist than ocelots, but may share similar environments, including overlapping home ranges (Leonard 2017). Bobcats and coyotes are more abundant than ocelots in Texas, and competition for prey may occur when prey are scarce (Tewes and Hornocker 2007).

NONINVASIVE GENETIC SAMPLING FROM SCAT

Serious pitfalls in noninvasive genetic studies can occur as a result of poor-quality DNA, since DNA degrades over time in the environment from UV exposure, heat, and moisture (Taberlet et al. 1999). Amplification of DNA from low-quantity and low-quality samples can lead to misidentification of genotyped individuals or amplification failure; it is important to collect and preserve samples properly (Foran et al. 1997). Contamination can occur during any step of the field or laboratory process by contact with cells from other organisms. Also, extractions from noninvasive samples can contain PCR inhibitors, substances that prevent amplification of DNA present in the sample (Taberlet et al. 1999, Farrell et al. 2000). Therefore, the use of noninvasive samples presents challenges in sample viability in the environment.

Scat and hair are often the most common noninvasive source of animal DNA (Foran et al. 1997). When comparing hair and scat collection methods, Ruell and Crooks (2007) found that 78% of the hair samples collected by their snares were non-felid, and their genotyping success from bobcat hairs was only 10% compared to 87% from bobcat scats. The extraction and amplification of DNA can be more successful when using bobcat scat as opposed to hair because felid hair is thin, making it more difficult to extract DNA compared to hair from other species (Ruell and Crooks 2007). The fine structure of felid hair could contain less genetic material than a coarse hair, which has more surface area for DNA carrying tissue to adhere to (Kendall and McKelvey 2008). Therefore, scat sampling can be advantageous over using hair in bobcat genetic research.

Carnivore scat contains DNA from cells of the defecating individual, prey items, and gut microbes. The extraction process elutes strands of DNA from biological products in the scat sample and stores them in a buffer solution. The highest concentration of DNA in the scat from the defecating individual is from epithelial cells remaining on the surface, which are sloughed from the colon wall as the scat is deposited (Reed et al. 2004, Rutledge et al. 2009). Scat contains

a low concentration of DNA compared to other sources such as blood, tissue, and feathers. The first step after finding a carnivore scat in the field is identifying which species deposited the sample. When accuracy of visual species identification is uncertain, generic primers can be used to amplify target fragments of DNA from the extracted solution, and the sequences can be aligned to references to identify species. Target sequences < 300 base pairs increases amplification success when using scat samples due to low quality and concentration of template DNA (Frantzen et al. 1998).

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CHAPTER II

PILOT STUDIES: NONINVASIVE GENTIC SAMPLING OF CARNIVORE SCAT

Noninvasive genetic sampling has become an increasingly effective tool for wildlife management, aided by advancements in laboratory methods and DNA sequencing technology. However, there is no consensus for optimal sample collection, preservation, and processing protocols (Waits and Paetkau 2005). Protocols can yield different success rates depending on environmental and laboratory conditions; pilot studies are often critical to adapt protocols for use in different settings (Panasci et al. 2011).

Studies that use animal scat collected in the field often rely on accurate identification of the species that deposited the sample. Field guides and experience aid identification, but environmental conditions and overlapping diets can increase the difficulty, particularly between feline and canine scat (Murie and Elbroch 2005). Some evaluations of experienced naturalists show visual species identification error rates as poor as 50% (Halfpenny 1986). However, the additional step of mitochondrial DNA sequencing to genetically confirm species can be time consuming, expensive, and inefficient if samples from non-target species are not used in further analyses. In a study where carnivore scats were collected along roads and trails in California, only about 10% tested were from bobcats (Ruell et al. 2009). It is unclear if visual identification can be accurate enough to avoid the added cost of processing non-felid samples.

In noninvasive genetic mark-recapture studies, many study designs incorporate multiple days between sampling occasions for accumulation of scats along transects (Ruell et al. 2009, Gulsby et al. 2016). Allowing scats to accumulate in a genetic mark-recapture study can increase detection probability between sampling occasions. However, in southern Texas, the abundance of dung beetles could make assumptions of sample persistence invalid, and make identifying features of the scat indiscernible. Emmons (1987) reported that ocelot scats only persisted more

Chapter II of this thesis is written in the style of *The Journal of Wildlife Management.*

than a few days when deposited on dry beaches, while dung beetles and trigonid bees removed scats within hours when deposited in forests. Additionally, the effect of insect scavenging on amplification of DNA is unknown.

Sampling methods typically target fresh scat samples that contain the highest quantity and quality of DNA, which degrades over time without proper preservation (Lucchini et al. 2002, Piggott 2004). Carnivore scats typically turn white as they dry, so white scats are avoided because of concerns regarding sample age and environmental exposure on DNA quality (Smith et al. 2003, Gosselin et al. 2017). However, the rate at which the scats turn from brown to white could vary across species and regional climates because of different desiccation rates. No studies for determining "freshness" or desiccation rates of carnivore scats by physical characteristics have been done in South Texas. Many studies that quantify DNA degradation rates in scat are uncertain of the age of the sample, and rely on appearance to categorize age classes (Panasci et al. 2011). Smith et al. (2003) observed that scat from kit foxes (*Vulpes macrotis mutica*) did not turn prominently gray or white until a 16-day period of exposure in the semi-arid summer in California. Previous studies suggest that scats > 1 week old will not efficiently produce reliable microsatellite genotypes (Piggott 2004). Harrison (2006) found that 41% of the bobcat scats collected and genetically confirmed were light grey or white, but also that most samples that failed to amplify were white. Thus, it is unclear if avoiding light grey or white scat in noninvasive genetic research will negatively bias species detections, or if light coloration is a valid indicator of sample age for bobcat scat.

The objectives of this study using were to: (1) optimize protocols for sample collection, preservation, and DNA extraction to enable species identification from mitochondrial DNA sequencing, and amplification of nuclear DNA for microsatellite genotyping, (2) determine if species of scat sample could be distinguished without the need for genetic confirmation, (3) determine how long a scat sample could persist in the environment and maintain amplifiable

DNA in the presernce of dung beetles, and (4) determine if white bobcat scat contains amplifiable mitochondrial and nuclear DNA.

STUDY AREA

I conducted this research on public and private land in San Patricio, Kenedy, Willacy, Cameron, and Hidalgo counties, Texas. The study areas are in South Texas within the Tamulipan Biotic Provence (Blair 1950). The Tamaulipan thornshrub ecosystem is unique to South Texas and northeast Mexico, and is characterized by dense thorny brush. This region has hot summers, with daily high temperatures in the upper 30s (°C), and mild winters that frequently remain above freezing (Haines et al. 2006). Annual precipitation averages 64 cm, but is highly variable across years (Laack 1991); average yearly relative humidity is about 76%. The study areas were managed for cattle ranching, wildlife, or both.

METHODS

Evaluating Protocols: Preservation, Extraction, Species Identification, and Genotyping

Sample Preservation.— I evaluated 2 principal methods of scat collection and preservation, including a buffer and frozen storage. I used the "freezing" method for samples collected between 9 December 2015 and 3 August 2016. I collected whole scats and avoided cross-contamination by wearing disposable latex gloves and inverting a plastic bag or 532 mL (18 oz) whirl-pak (Nasco, Stamford, CT). I added 1-3 5-g absorbent packets of MiniPax® silica gel based on the moisture of the sample to remove moisture and prevent molding and degradation of the sample. Samples were kept in a shaded compartment in the vehicle and transported to a -10 °C mini-freezer within 4 hr from the time of collection. Samples were kept in

the mini-freezer between 0.5-8 days before being transported to a -20 °C freezer. Samples were kept at -20 °C until immediately prior to extraction.

The "DETs buffer" method was used for samples collected between 21 October 2016 and 19 May 2017. I placed 0.4 mL of the outer surface of each scat into 2-mL screw top tubes containing 1.4 mL of DETs (DMSO/EDTA/Tris/salt) buffer (Frantzen et al. 1998). The DETs buffer consists of 20% dimethyl sulfoxide, 0.25M sodium-EDTA, 100 mM Tris, pH 7.5, and NaCl to saturation (Seutin et al. 1991). Samples were kept in a portable cooler while in the field and stored at 4 °C within 8 hr of collection. For each sampling occasion, I wore disposable latex gloves and changed gloves between samples.

DNA extraction.— I performed DNA extractions under a fume-hood in the Molecular Ecology Laboratory at Texas A&M University–Kingsville. Each frozen sample was taken individually from the -20 °C storage and placed on a sterile disposable petri dish in the fume-hood. Forceps were cleaned with 95% ethanol prior to use for handling each sample. I shaved the surface of each frozen scat with a new razor blade to target epithelial cells remaining on the surface from the colon wall of the defecating animal (Reed et al. 2004, Rutledge et al. 2009). Enough scat was used to displace 400 µL of lysis buffer. For the DETs method, I began by homogenizing the samples in the 2 mL collection tubes of DETs buffer for 3 min using a vortex mixer. I pipetted 200 µL of homogenized scat into the tube of lysis buffer by cutting the end of a 1,000-µL filtered pipette tip. For the frozen and DETs methods, I used the QIAamp® DNA Stool Mini Kit and the protocol recommended by the manufacturer (Qiagen, Hilden, Germany), with the exception that I performed the final elution step using 120 µL of storage buffer after incubation for 5 min. I performed extractions in sets of 12 with 11 samples and 1 negative, or in sets of 24 with 23 samples and 1 negative. Extraction negatives contain only reagents but are

treated as a sample during the extraction process for quality control (Waits and Paetkau 2005). I stored DNA extracts at 4º C until further use.

Species Identification.— I performed mitochondrial DNA sequencing to identify species for each scat sample. I tested 3 sets of primers tested by Chaves et al. (2012) that distinguished among 66 species of carnivores, including 12 felids, without amplifying non-carnivore species of prey. I selected primer sets to sequence mitochondrial DNA fragments from genes ATP6, Cytochrome c oxidase I (COI), and Cytochrome b (Cyt-*b)* (Table 2.1; Chaves et al. 2012). I selected 8 samples to independently sequence with each primer set. These 8 samples included 2 ocelot blood and scat positives, 2 bobcat blood and scat positives, and 4 unknown scat samples. The 8 samples were from 6 independent extractions. Positive samples were collected from bobcats and ocelots trapped during a separate study. The 25-µL PCR volume for each sample contained 12.5 µL of AmpliTaq Gold® 360 PCR master mix (Taq DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers; Applied Biosystems, Foster City, CA), 8.3 µL double-deionized H_2O, 1 µL bovine serum albumin (BSA; 2 mg/mL; Thermo Scientific, Waltham, MA), 0.1 µL of each forward and reverse primer at 100 µM (10 pmol), and 3 µL DNA extract. Each PCR included an extraction negative and known positive from blood or scat of known individual bobcats or ocelots previously trapped in the area.

The thermocycling conditions to amplify ATP6 were an initial denaturation at 94 °C for 3 min, followed by 10 cycles at 94 °C for 45 sec, a touchdown annealing temperature of 60 °C for 45 sec that decreased 1 °C each cycle to 50 °C, 72 °C for 1 min 30 sec, followed by 30 cycles of 94 °C for 45 sec, 50 °C for 45 sec, 72 °C for 1 min 30 sec, and a final extension at 72 °C for 5 min. I tested an additional 18 samples using a 3-min initial denaturation instead of 10 min to allow the enzyme activity from the built-in hot start polymerase to increase over cycles as the amount of template increased (Lorenz 2012, Applied Biosystems).

The thermocycling conditions for COI were an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 sec, a within-cycle decreasing annealing temperature of 50 °C for 20 sec, 48 °C for 5 sec, 46 °C for 5 sec, 44 °C for 5 sec, 42 °C for 5 sec, 40 °C for 20 sec, and extension of 72 °C for 1 min 30 sec, with a final extension at 72 °C for 5 min.

The thermocycling conditions for Cyt-*b* were an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 sec, 50 °C for 45 sec, and a final extension of 72 °C for 40 sec followed by a final extension at 72 °C for 5 min.

I visualized PCR products with electrophoresis on a 1% agarose gel containing ethidium bromide and a 100 base-pair DNA ladder. Samples that did not show a band of the predicted size were considered a failed amplification and removed from further analysis. I removed unincorporated primers and dNTPs from successful amplifications using an enzymatic method (ExoSAP-IT®, Affymetrix Inc., Santa Clara, CA). I sequenced forward strands using the BigDye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems), and removed dye terminators using a DyeEx® 2.0 spin kit (Qiagen). I mixed sequencing reaction products with 13 µL of Hi-Di formamide for denaturing, and loaded a 3130xl Genetic Analyzer (Applied Biosystems). I edited and aligned sequences using the program Geneious® V10 (Kearse et al. 2012). I compared the sequences to references available in Genbank using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990). The sequencing of the sample was considered successful in this analysis if I could determine species using a consensus of the top 3 results BLAST.

Microsatellite Genotyping.— I initially tested PCR protocols for nuclear DNA by comparing amplification rates from 8 bobcat samples using 2 blood and 6 scat DNA extracts. Each 10 µL PCR contained 5 µL of AmpliTaq Gold® PCR master mix (Applied Biosystems), 1.8 µL double-deionized H_2O, 0.2 µL BSA (20 mg/µL, Thermo Scientific), 0.5 µL of each forward

and reverse primer at 10 μM, and 2 μL DNA extract. Thermocycling conditions were an initial denaturation at 94 °C for 10 min, followed by 10 cycles at 94 °C for 30 sec, a within-cycle decreasing annealing temperature of 62 °C for 30 sec, 61 °C for 30 sec, 60 °C for 30 sec, and an extension of 72 °C for 60 sec, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 90 sec, and 72 °C for 60 sec, and a final extension at 72 °C for 30 min. I amplified 11 microsatellite loci in separate reactions, then combined reaction products into 3 panels for separation and detection. Panel 1 was a mixture of 2 μL FCA008, 1 μL FCA043, 1 μL FCA082, 2 μL FCA090 and 5 μL double-deionized H_2O. Panel 2 was a mixture of 2 μL FCA035, 2 μL FCA096, 1 μL FCA132, 1 μL FCA133, and 5 μL double-deionized H_2O. Panel 3 was a mixture of 1 μL each FCA045, FCA077, FCA176 and 5 μL double-deionized H_2O. Markers were originally described by Menotti-Raymond et al. (1999). I denatured 1.1 μL of each diluted PCR product mixture with 0.5 μL GeneScan ROX500 size standard and 11 μL Hi-Di formamide (Applied Biosystems). The resulting product was run on an Applied Biosystems 3130*xl* genetic analyzer for fragment separation and detection. I scored alleles using the computer program Genemapper® (Applied Biosystems). I calculated failed amplification rates instead of success rates in nuclear DNA analyses because amplification "success" can be misleading if genotyping errors such as allelic dropout or false alleles are not accounted for. A failed amplification is defined as the number of individual PCRs with the absence of a scored allele within the correct size range for each locus divided by the number of individual PCRs attempted at each locus (Panasci et al. 2011).

I attempted to improve amplification rates from 7 failed samples by testing 4 different ratios and volumes of reagents, as well as a pre-amp 2-step PCR developed by Piggott et al. (2004) to amplify DNA from low quantities. I also tried to purify PCR product using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany), and remove PCR inhibitors using a Zymo OneStep PCR Inhibitor Removal kit (Zymo Research, Irvine, CA).

Visual species identification of bobcat and coyote scat

Seven wildlife professionals with prior exposure to scat identification were given 10 digital photographs of scat and asked to choose between bobcat *(Lynx rufus)* and coyote *(Canis latrans)*. I used photographs of scat that I collected from the Welder Wildlife Refuge in Sinton, Texas. No vegetation or seeds were present in any of the samples, which suggested dietary overlap of coyotes and bobcats during the winter in South Texas. The images and positions of the scats were unaltered, and included an object next to the scat for a visual size reference. Their responses were recorded and the scats were confirmed genetically using the frozen scat protocol and mitochondrial DNA sequencing. Additionally, I recorded my species predictions while collecting scats for concurrent research, and 251 of those were genetically identified to species. I separated my accuracy into 3 sessions to calculate my performance after learning from photographs of scats and laboratory results between each session.

Sample persistence in the environment

I established transects on ranch roads and cleared carnivore scat 1 day prior to sampling to ensure samples were < 24-hr old for initial detection. I then identified 12 scats on day 1 and collected a small segment of each for storage until DNA extraction using the frozen scat protocol. The rest of the scat was left in the field at its original location, marked with flagging and GPS location, photographed each day, and collected again on day 5 for a separate DNA extraction. I recorded physical appearance if the scat remained, and determined if the sample contained amplifiable DNA based on success rates of mitochondrial DNA sequencing.

Amplification of DNA from white scats

I opportunistically collected white scats of unknown age while sampling along roads and trails. I also collected fresh scats < 24-hr old along transects surveyed daily. Scats were either collected using the freezing or DETs buffer storage method. I determined the amplification success and identified species using mitochondrial DNA sequencing. I determined amplification

success of nuclear DNA for bobcat samples using microsatellite loci genotyping by calculating failed amplification rates. I acquired consensus genotypes after independently amplifying loci at least twice and accepting 2 matching heterozygote and 3 matching homozygote alleles at each locus using the multiple tube approach (Taberlet et al. 1996). I quantified genotyping errors from loci with consensus genotypes by calculating mean allelic dropout and false allele rates (Broquet and Petit 2004). I randomly selected the same number of fresh and white scat samples for analysis as a balanced design.

RESULTS

Evaluating Protocols: Preservation, Extraction, Species Identification, and Genotyping

Sample Preservation and DNA Extraction.— I used the results of microsatellite genotyping from felid-specific primers for troubleshooting the first collection and extraction methods from whole frozen scats. However, amplification success was not significantly improved until using the DETs collection and extraction protocol (See results for "Microsatellite Genotyping").

Species Identification.— I successfully identified 75% (n = 8) of samples to species using primer sets from ATP6 and Cyt-*b,* and 12.5% (n = 8) from COI. Thus, COI had the lowest amplification success and was eliminated from further analysis. I also eliminated Cyt-*b* for amplifying a sequence that loosely matched thicket rats (*Grammomys* spp.) and red-backed voles (*Myodes* spp.), most likely a short fragment of prey DNA from a rodent in 1 of the samples. Therefore, I chose to use the ATP6 primers for the remainder of the study. Additionally, I successfully sequenced 72.2% (n = 18) using the 3-min initial denaturation PCR protocol and 22.2% (n = 18) using the 10-min protocol (Fig. 2.1). Thus, I chose to use a 3-min initial denaturation for the remainder of the study.

However, I identified contamination in PCR-negatives and extraction-negatives while sequencing additional samples. Multiple negatives containing only reagents and double-deionized H_2O revealed bands in the gel electrophoresis at the correct length. Some of these negative samples sequenced to a coyote or bobcat. I determined the source of contamination by using new aliquots of primers and reagents except for the source evaluated. I cleaned equipment with a bleach solution and set it under a UV light for over 1 hr before use. I identified the contamination was coming from a bottle of BSA since PCR products in the negative controls were visible in the gel until a new bottle of BSA was purchased. The contaminated bottle was initially opened 7 years ago under unknown conditions, and amplified product was no longer visible in the PCR and extraction negatives after using a new bottle of BSA.

Microsatellite Genotyping.— The PCR protocol had a failed amplification rate of 17% across 8 selected samples, including 2 blood and 6 scat extracts. All unamplified markers were from 2 of the scat samples, meaning 2 blood and 4 scat samples had alleles present in all 11 loci amplified. This is indicative of variation in the quality of individual samples, but the PCR protocol was successful for amplifying nuclear DNA from blood and scat extracts.

However, I began troubleshooting PCR and extraction protocols when the next 7 bobcat scat samples had an amplification success of 92.2%. The positive blood sample included in the same PCR had no failed amplifications to validate that there was no user-error in the PCR. None of the troubleshooting experiments that included different ratios and volumes of reagents, a pre-amp 2-step PCR, PCR product purification, and PCR inhibitor removal made any significant improvement in amplification success from these samples.

The 62 bobcat samples collected between 9 December 2015 and 3 August 2016 with the freezing method had a failed amplification rate of 53.1% (529/996 loci) with nuclear DNA markers. Of these, 36 samples considered fresh and < 24-hr old had a failed amplification rate of

66.9% (343/513 loci) and 26 samples collected opportunistically and > 1 day old had a failed amplification rate of 38.5% (186/483 loci).

Amplification success significantly improved with the DETs collection and extraction protocol. I collected 140 bobcat samples between 21 October 2016 and 19 May 2017 with the DETs buffer and had a failed amplification rate of 18.2% (638/3509 loci) with nuclear DNA markers. The DETs method lowered the failed amplification rate by 65.8% compared to using frozen samples. There was also improvement in signal strength on allele calls for the samples collected in the DETs buffer. The 3 PCR product mixtures had to be adjusted to diminish signal strength. Panel 1 was a mixture of 2.5 µL FCA008, 1 µL FCA043, 1.5 µL FCA082, 1 µL FCA090 and 20 µL double-deionized H_2O. Panel 2 was a mixture of 3.5 µL FCA035, 2.5 µL FCA096, 1 µL FCA132, 2 µL FCA133, and 20 µL double-deionized H_2O. Panel 3 was a mixture of 2 µL FCA045, 1.5 µL FCA077, 1 µL FCA176 and 20 µL double-deionized H_2O.

Variability in individual sample quality was still evident with the DETs preservation method. I re-extracted 6 scat samples from DETs buffer samples that sequenced as bobcat but had poor nuclear DNA amplification success with a failed amplification rate of 72% (95/132 loci) for 2 rounds of PCR. The re-extracted samples had the same failed amplification rate (72%, 39/54 loci) over 1 round of PCR.

Visual species identification of bobcat and coyote scat

Seven wildlife professionals correctly identified an average of 64% of the scat images (individuals range 50-90%). I was correct for 78.5% of 251 scats I collected in the field that were genetically confirmed. However, I was only targeting bobcat samples, so I was correct for 82.1% of 179 samples that I believed were from bobcats. In session 1, I collected 31 samples that I believed were bobcat without any prior field identification experience and was correct for 61.3% of scats. After learning from photographs and laboratory results of session 1, I collected 101 scats for session 2 that I believed were bobcat, and was correct for 78.2%. I improved 27.6%

between session 1 and 2. After learning from the laboratory results from session 1 and 2, I collected 47 scats for session 3 that I believed were bobcat and was correct for 100%. I improved 27.8% from session 2 to 3.

Sample persistence in the environment

The 12 scats collected on day 1 were indiscernible within 24 hr because of scavenging by dung beetles. Two scats were physically removed by day 2, and 1 by day 3, leaving 9 loose clusters of undigested prey remains collected on day 5. Mitochondrial DNA amplification for species identification was successful for 91.7% (n = 12) of samples collected on day 1 and 88.9% (n = 9) of samples collected on day 5.

Amplification of DNA from white scats

Amplifiable mitochondrial DNA was successfully sequenced from 82.5% (n = 40) of white scats compared to 92.5% (n = 40) of fresh scats. Of the white scats, 77.8% (n = 18) and 86.4% (n = 22) sequenced from frozen and DETs, respectively. Of the fresh scats, 83.3% (n = 18) and 100% (n = 22) sequenced from frozen and DETs, respectively.

I used the 30 white bobcat scats and 30 fresh bobcat scats that were successfully sequenced for further analysis. Failed amplification rate of white scat was 27.8% compared to 35.0% of fresh scat. When frozen, failed amplification rate was 42.2% for white scat and 79.2% for fresh scat. When in DETs buffer, failed amplification rate was 21.9% for white scat and 19.9% for fresh scat. Allelic dropout rate of white scat was 11.4% compared to 14.5% of fresh scat. False allele rate was 4.8% for white scat compared to 4.8% for fresh scat.

DISCUSSION

Evaluating Protocols: Preservation, Extraction, Species Identification, and Genotyping

The ATP6 primers performed best for this study, which is consistent with Chaves et al. (2012) that found these ATP6 primers have the most amplification success with high

discriminatory power and no amplifications of prey DNA. Although mitochondrial DNA amplification was not significantly affected by sample preservation and extraction method, the DETs buffer performed significantly better than the frozen scat method. Because failed amplifications were significantly less for > 1 day old scat than fresh scat when using the freezing storage technique, this could support the importance of desiccation and proper freezing temperature when preserving DNA without a buffer. Scats were not placed in silica long enough before freezing to completely desiccate as they would over time in the environment. A study by Brinkman et al. (2010) found high amplification success from deer scat samples up to 28 days in a dry environment. However, rainfall significantly decreased success, which also indicates the compounding effect of moisture on DNA degradation in scat (Brinkman et al. 2010). Although studies show fresh scat to contain the highest quality DNA for amplification (Lucchini et al. 2002, Piggott 2004), rapid degradation likely occurred in preservation and storage protocol. Therefore, I recommend using a DETs buffer for noninvasive genetic sampling.

Re-extracting did not improve amplification rates for microsatellite genotyping, therefore, extractions were consistent and not the source of variability in sample quality. Factors affecting inherent variation in sample quality could be related to diet composition (Panasci et al. 2011), contamination from other sources of DNA (Broquet et al. 2007), environmental factors such as sun exposure (Farrell et al. 2000), or other unknown sources. Although some studies report the presence of PCR inhibitors found in animal tissues in carnivore scat (Murphy et al. 2003), inhibitors were not prevalent in our extracted DNA because the purification and inhibitor removal kits did not increase amplification success.

Visual species identification of bobcat and coyote scat

Wildlife professionals were unable to reliably distinguish between bobcat and ocelot scat from photographs. Accuracy was expected to be lower than a field test because a photograph takes away the ability to observe contextual clues, scent, and texture. However, most observers

rely on field guides, which lack the same advantages for aiding scat identification. Although these results are not meant to be a precise measure of accuracy, they provide insight for the difficulty of scat identification in South Texas when coyote and bobcat diets overlap.

Although I eventually achieved 100% accuracy after my experience of collecting and analyzing > 200 scats, I believe my overall accuracy around 80% is a more accurate measure of visual species identification success. One reason is that potential bias exists in seasonal differences in diet composition. Coyote diets change seasonally, so indicators such as the presence of seeds could affect identification of bobcat scats. Session 1 occurred between 17 December 2015 and 4 May 2016. Session 2 occurred between 3 August 2016 and 21 January 2017, with all but 6 samples collected in December or January. Session 3 occurred in May between 15 May 2017 and 19 May 2017, and the presence of seeds in coyote scats during this time could have made it easier to identify bobcat scat. Additionally, accuracy of identifying bobcat scat may decrease in other regions where different carnivores are present. Therefore, I suggest that visual identification should be followed by genetic confirmation in studies that depend on accurate species identification.

Sample persistence in the environment

Dung beetles arrived and began scavenging on all scats within 24 hr of defecation, and remains of 3 scats could not be found after 1 or 2 days. This observation suggests 25% of scats would not have been detected with a sample accumulation period > 2 days. The scats scavenged by dung beetles were indiscernible clumps of hair that would make visual species identification difficult. There was no significant difference between mitochondrial DNA amplification success of samples collected on day 1 compared to samples collected on day 5. This suggests that mitochondrial DNA has potential to persist in the environment for at least 4 days after insect scavenging. Therefore, scavenging by dung beetles did not correlate with mitochondrial DNA amplification success, but is an important factor when considering field identification and

persistence of scat. Competition with scavenging insects should be considered when designing a study using scat surveys.

Amplification of DNA from white scats

I amplified mitochondrial DNA from 83% of carnivore scats collected with a beached-white appearance. Mitochondrial DNA sequencing success was 10% lower for white scats compared to fresh scats, but was still relatively high given the possibility for a wide range of sample age and exposure. This result suggests that white coloration is not a good indicator of mitochondrial DNA amplification success for short fragments of DNA.

White scat contained amplifiable nuclear DNA. Overall, there was no difference in failed amplification from white scat compared to fresh scat, which was true when storing scat in a DETs buffer. However, failed amplifications were significantly less for white scat than fresh scat when using the freezing storage technique, which provides additional support for the importance of desiccation and proper freezing temperature when preserving DNA without a buffer.

There was no significant difference in genotyping error rates of white scat compared to fresh scat. Because the white scats were not prone to more errors than fresh scat, the white scats may not necessarily be older than a few days. For comparison, I used the same calculations for allelic dropout, false allele, and failed amplification rates as Panasci et al. (2011). They used aged coyote scats and found that from day 1 to 5, allelic dropout rates increased from 18% to 38%, false allele rates increased from 21% to 36%, and failed amplification rates increased from 6% to 11% (Panasci et al. 2011). This trend in increasing error rates was not linear and there was no significance between day 5 and 10, suggesting most degradation occurs within the first 5 days of sample exposure (Panasci et al. 2011). Additionally, if Piggot (2004) found that scat > 7-10 days old produced unreliable genotypes, the white scats I collected may be < 1 week old. I was unable to determine sample age of the white scats I collected, but Smith et al. (2003) reported kit fox scats remaining brown after 8 days, and were only developing “small white marks” between

16 and 32 days. Bobcat scats could turn white faster compared to other species or as a factor of exposure to the hot and humid environment of South Texas. Therefore, white coloration is not a good indication of sample age in the South Texas environment.

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Table 2.1. Primer sequences tested to amplify mitochondrial DNA for species identification from carnivore scat collected in Texas, USA, during 2015–2017 (Chaves et al. 2012). Sequence lengths do not include the forward and reverse primers.

Gene	Primer sequence 5′- 3′	Source	Sequence length
ATP6-DF3	AACGAAAATCTATTCGCCTCT	Haag et al. 2009	126 bp
ATP6-DR1	CCAGTATTTGTTTTGATGTTAGTTG	Trigo et al. 2008	
COI-BC-F3	CCCCTATTCGTATGATCAGTATTAATTAC	Chaves et al. 2012	184 bp
COI-BC-R2	TAAACCTCAGGATGTCCGAAGAATCA	Chaves et al. 2012	
Cytochrome *b*-F	AAACTGCAGCCCCTCAGAATGATATTTG TCCTCA	Farrell et al. 2000	110 bp
Cytochrome *b*-R	TATTCTTTATCTGCCTATACATRCACG	Farrell et al. 2000	

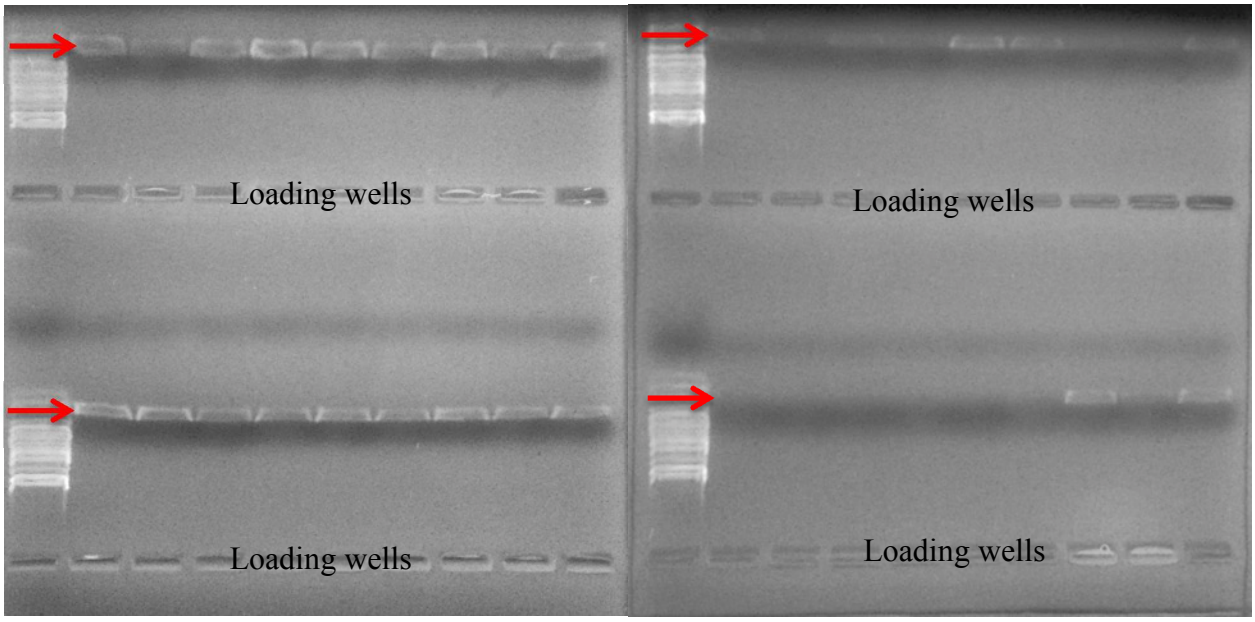

Figure 2.1. Agarose gel electrophoresis with ethidium bromide stain visualizing PCR products from DNA of the same 18 scat samples collected in Texas, USA, during 2015–2017. I used 2 protocols that differ by a 3 min or 10 min initial denaturation. Positive amplifications are a 172 base-pair fragment of the ATP6 gene shown as a bright white band horizontal to the red arrows.

CHAPTER III

EVALUATION OF NONINVASIVE SCAT SAMPLING FOR GENETIC MONITORING OF BOBCATS AND OCELOTS IN SOUTH TEXAS

The historic range of the ocelot (*Leopardus pardalis*) in the U.S. extended into parts of Arizona, Arkansas, Louisiana, and Texas (Woodward 1980). However, habitat loss during the 20th century caused significant declines in ocelot populations because of direct take and the removal of thornshrub and dense woody canopy habitats (Tewes and Everett 1986). The ocelot is currently listed as endangered in the U.S. (U.S. Department of Interior, Fish and Wildlife Service, 1982). Fewer than 80 ocelots remain in South Texas, with only 2 known breeding populations (Tewes and Everett 1986, Haines et al. 2005, Tewes 2017). The "Cameron population" occurs in Cameron County on and around the Laguna Atascosa National Wildlife Refuge (LANWR), and typically consists of about 10-15 individuals (Tewes 2017). The "Willacy population" occurs about 30 km to the northwest on private lands in Willacy and Kenedy counties, and consists of over 40 individuals (M. E. Tewes, Texas A&M University-Kingsville, personal communication; Tewes 2017).

Some of the highest rates of human population growth and development in the U.S. occur in the Lower Rio Grande Valley (LRGV) of Texas (Fulbright and Bryant 2002, Leslie 2016). Vehicle-caused mortality accounts for 35% of ocelot deaths in the remnant Texas populations (Haines et al. 2005). The U.S. Fish and Wildlife Service reported 7 road-killed ocelots in < 11 months during 2015 and 2016 (U.S. Fish and Wildlife Service, unpublished data). Loss of genetic diversity in the 2 remaining populations due to genetic drift is an additional concern for the long-term viability of ocelots in the U.S. (Janecka et al. 2008, 2011). Although the Cameron and Willacy populations are < 30 km apart, they are demographically isolated from each other and from source populations in Mexico (Haines et al. 2006, Tewes 2017). Future translocation or

Chapter III of this thesis is written in the style of *The Journal of Wildlife Management.*

assisted reproduction with ocelots from northern Mexico is necessary to restore genetic diversity (Haines et al. 2006).

Population monitoring and successful translocations rely on genetic sampling from the remaining ocelots in the U.S. (Weeks et al. 2011). Current efforts to monitor this rare and elusive species include live-trapping and camera detection stations. Trapping efforts sometimes result in the capture of only a few individuals. Few research scientists can attain permission to trap because most ocelots inhabit private lands. In contrast, camera traps yield more detections, and individuals can be identified using their unique spotting patterns. However, camera traps lack the ability to gather genetic information. Thus, additional survey tools are needed to aid in conservation efforts for the remaining ocelots in the U.S.

Ocelots in Texas occupy dense thornshrub (Shindle and Tewes 1998, Horne et al. 2009), with almost completely closed canopy (Tewes 1986, Laack 1991) often inaccessible by humans. Observations from game cameras, satellite collars, and trapping locations show that ocelots make forays into adjacent open areas, such as ranch roads or trails (Laack 1991, personal obs.). Many wild felids and other carnivores are known to defecate along roads and trails for communication and territorial marking (MacDonald 1980, Kohn et al. 1999). In particular, bobcats (*Lynx rufus*) occur sympatrically with the Willacy and Cameron populations of ocelots. Bobcats are less of a habitat specialist than ocelots (Tewes and Schmidly 1987, Horne et al. 2009), but share similar environments, including overlapping home ranges (Leonard 2017). It is unclear if bobcat abundance affects ocelots through interspecific competition, so it is important to monitor niche partitioning for management of both felids (Leonard 2017). Scats can serve as a noninvasive source of DNA from the defecating individual (Höss et al. 1992). Thus, it may be possible to incorporate noninvasive scat sampling along roads and trails as an additional monitoring tool for ocelots and bobcats.

Objectives of this research were to: (1) detect presence of ocelots and bobcats using noninvasive fecal collections along ranch roads, (2) estimate abundance of ocelots and bobcats using genetic capture-recapture, and (3) determine if the additional effort invested in noninvasive scat sampling in conjunction with camera and live-trapping provides a useful contribution as a monitoring strategy for ocelots and bobcats in South Texas.

STUDY AREA

I conducted research on the East Foundation's El Sauz Ranch, located on 110 km^2 in Willacy and Kenedy counties, Texas (Fig. 3.1). The eastern border of the property is the coastline of the Laguna Madre of the Gulf of Mexico, and the western portion of the property includes known ocelot habitat. The study area is located in South Texas within the Tamaulipan Biotic Provence (Blair 1950). This region has hot summers, with daily high temperatures in the upper 30s (°C) and mild winters (Haines et al. 2006). Annual precipitation averages 64 cm, but is highly variable across years (Laack 1991). The major soil associations are sandy, with sand dunes in the central part of the ranch adding topography to an otherwise flat landscape. The Tamaulipan thornshrub ecosystem is unique to South Texas and northeast Mexico and is characterized by dense thorny brush (Blair 1950). Woody habitat that provides canopy cover preferred by ocelots (Shindle and Tewes 1998, Horne et al. 2009) and bobcats (Cain et al. 2003) is dominated by native thornshrub, honey mesquite *(Prosopis glandulosa),* and live oak *(Quercus virginiana)* communities. The study area was managed for cattle ranching and wildlife; the predominant land use in the region consisted of cattle ranching, agriculture, energy development, and urbanization.

METHODS

Sample Collection

I established 34 km of transects using existing ranch roads in the northwest and southwest areas of the ranch, where ocelots were known to occur (Fig. 3.2). Road substrate consisted of either crushed caliche stone or sandy 2-track. I estimated effective sampling area in ArcGIS by placing a 2 km^2 buffer around the transects (Fig. 3.3), which is the smallest ocelot home-range size reported for a female ocelot in South Texas (Navarro-Lopez 1985, Tewes 1986, Laack 1991). I removed carnivore scats from the transects 1 day prior to the sampling period to avoid collecting a scat that was not deposited during the sampling period. Sampling occasions began every morning at sunrise the following day after clearing. I collected carnivore scats that were potentially from an ocelot or bobcat, and collected scats if identification was uncertain to avoid missing detections. Collections were repeated for 14 days in spring 2016 and 10 days in winter 2017, using short sampling periods to maximize the chance of a relatively closed population for estimating abundance (Miller et al. 2005). I photographed and recorded GPS location for each scat collected.

The DNA in scat will degrade over time, so it was important to collect and quickly preserve samples (Foran et al. 1997). I used 2 principal methods of scat preservation, freezing and a buffer. During 2016, I collected whole scats by inverting a plastic bag or 532 mL whirl-pak (Nasco, Stamford, CT), and added 1-3 5-g absorbent packets of MiniPax® silica gel (Sigma-Aldrich, St. Louis, MO) to remove moisture and prevent molding and degradation of the sample. Samples were kept in a shaded compartment in the vehicle and transported to a cooler with dry ice within 4 hr from the time of collection. Samples were kept in the cooler with dry ice between 2-8 days before being transported to a -20 °C freezer. Samples were kept at -20 °C until immediately prior to extraction.

During 2017, I placed 0.4 mL of the outer surface of each scat into 2-mL screw top tubes containing 1.4 mL of DETs (DMSO/EDTA/Tris/salt) buffer (Frantzen et al. 1998). The DETs buffer consists of 20% dimethyl sulfoxide, 0.25 M sodium-EDTA, 100 mM Tris, pH 7.5, and NaCl to saturation (Seutin et al. 1991). Samples were kept in a portable cooler while in the field and stored at 4 °C within 8 hr of collection. I wore disposable latex gloves and changed gloves between samples.

DNA extraction

I performed DNA extractions under a fume-hood in the Molecular Ecology Laboratory at Texas A&M University–Kingsville. Each frozen sample was taken individually from the -20 °C storage and placed on a sterile disposable petri dish in the fume-hood. Forceps were cleaned with 95% ethanol prior to use for handling each sample. The surface of each frozen scat was shaved with a new razor blade to target epithelial cells remaining on the surface from the colon wall of the defecating animal (Reed et al. 2004, Rutledge et al. 2009). Enough scat was used to displace 400 µL of lysis buffer. For the DETs method, I began by homogenizing the samples in the 2 mL collection tubes of DETs buffer for 3 min using a vortex mixer. I pipetted 200 µL of homogenized scat into the tube of lysis buffer by cutting the end of a 1,000-µL filtered pipette tip. For both methods, I used the QIAamp® DNA Stool Mini Kit and the protocol recommended by the manufacturer (Qiagen, Hilden, Germany), with the exception that I performed the final elution step using 120 µL of storage buffer after incubation for 5 min. I performed extractions in sets of 12, including 11 samples and 1 extraction negative of reagents for quality control (Waits and Paetkau 2005). I stored DNA extracts at 4º C until further use.

Species Identification

I sequenced the mitochondrial DNA ATP6 gene to identify species for each scat sample. I chose primers tested by Chaves et al. (2012) that distinguished between 66 species of carnivores, including 12 felids, without amplifying non-carnivore species of prey. I also

confirmed the ability of the primers to identify bobcat, ocelot, jaguarundi *(Puma yagouaroundi)*, and mountain lion *(P. concolor)* from DNA extracted from the blood of known individuals. I included jaguarundi and mountain lion in testing distinguishability because they have been historically documented in the area. I determined the number of single-nucleotide polymorphisms (variable sites) between sequences of 20 bobcats, 5 ocelots, 1 jaguarundi, and 2 mountain lions using the computer program Geneious® V10 (Kearse et al. 2012). I constructed a percent identity matrix in Geneious® V10 to quantify variability among sequences. Each PCR included an extraction negative and known positive. The fragments were 172 base-pairs total, with 126 base-pairs excluding forward and reverse primers (Chaves et al. 2012).

- ATP6-DF3 (L7987) 5′–AACGAAAATCTATTCGCCTCT–3′ (Haag et al. 2009).
- ATP6-DR1 (H8114) 5′–CCAGTATTTGTTTTGATGTTAGTTG–3′(Trigo et al. 2008).

The 25-µL PCR for each sample contained 12.5 µL of AmpliTaq Gold® 360 PCR master mix (*Taq* DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers; Applied Biosystems, Foster City, CA), 8.3 µL double-deionized H_2O, 1 µL bovine serum albumin (BSA; 2 mg/mL; Thermo Scientific, Waltham, MA), 0.1 µL of each forward and reverse primer at 100 µM (10 pmol), and 3 µL DNA extract. Thermocycling conditions were an initial denaturation at 94 °C for 3 min, followed by 10 cycles at 94 °C for 45 sec, a touchdown annealing temperature of 60 °C for 45 sec that decreased 1 °C each cycle to 50 °C, 72 °C for 1 min 30 sec. Next, 30 cycles of 94 °C for 45 sec, 50 °C for 45 sec, and 72 °C for 1 min 30 sec were followed by a final extension at 72 °C for 5 min. I chose to use 3 min initial denaturation instead of 10 min to allow the enzyme activity from the built-in hot start polymerase to increase over cycles as the amount of template increased (Lorenz 2012, Applied Biosystems).

I visualized PCR products with electrophoresis on a 1% agarose gel containing ethidium bromide and a 100 base-pair DNA ladder. Samples that did not show a band of the predicted size were removed from further analysis. I removed unincorporated primers and dNTPs from

successful amplifications using an enzymatic method (ExoSAP-IT®, Affymetrix Inc., Santa Clara, CA). I cycle-sequenced forward strands using the BigDye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems), and removed dye terminators using a DyeEx® 2.0 spin kit (Qiagen). I mixed sequencing reaction products with 13 µL of Hi-Di formamide for denaturing, and loaded the mixtures onto a 3130*xl* Genetic Analyzer (Applied Biosystems). I edited and aligned sequences using the program Geneious® V10 (Kearse et al. 2012). I compared the sequences to references available in Genbank using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990). I determined species using a consensus of the top 3 results. Positive samples from trapped bobcat and ocelot sequences were also used for reference. Sequences that aligned with non-target species were removed from further analysis.

Individual Identification

Individual bobcats and ocelots were identified using microsatellite genotyping. The empirically optimized protocol for a 10 µL PCR contained 5 µL of AmpliTaq Gold® PCR master mix (Applied Biosystems), 1.8 µL double-deionized H_2O, 0.2 µL BSA (20 mg/µL), 0.5 µL of each forward and reverse primer at 10 µM, and 2 µL DNA extract. Thermocycling conditions for were an initial denaturation at 94 °C for 10 min, followed by 10 cycles at 94 °C for 30 sec, a within-cycle decreasing annealing temperature of 62 °C for 30 sec, 61 °C for 30 sec, 60 °C for 30 sec, and an extension of 72 °C for 60 sec. Next, 40 cycles at 94 °C for 30 sec, 55 °C for 90 sec, and 72 °C for 60 sec were followed by a final extension of 72 °C for 30 min.

Eleven microsatellite loci were amplified in separate reactions; the reaction products were then combined into 3 panels for separation and detection. Markers were originally described by Menotti-Raymond et al. (1999), and these 11 loci were chosen based on previous studies of bobcats ocelots in Texas (Janecka et al. 2008, 2016; Davis 2015). Panel 1 was a mixture of the following reaction products 2 µL FCA008, 1 µL FCA043, 1 µL FCA082, 2 µL FCA090 and 5 µL double-deionized H_2O. Panel 2 was a mixture of 2 µL FCA035, 2 µL

FCA096, 1 µL FCA132, 1 µL FCA133, and 5 µL double-deionized H_2O. Panel 3 was a mixture of 1 µL each FCA045, FCA077, FCA176 and 5 µL double-deionized H_2O. I denatured 1.1 µL of each diluted PCR product mixture with 0.5 µL GeneScan ROX500 size standard and 11 µL Hi-Di formamide (Applied Biosystems). The resulting product was run on an Applied Biosystems 3130*xl* genetic analyzer for fragment separation and detection.

I scored alleles and merged into consensus genotypes using the computer program Genemapper® (Applied Biosystems). I calculated failed amplification rates instead of success rates in nuclear DNA analyses because amplification "success" can be misleading in the presence of genotyping errors such as allelic dropout or false alleles. A failed amplification is defined as the number of individual PCRs with the absence of a scored allele within the correct size range for each locus divided by the number of individual PCRs attempted at each locus (Panasci et al. 2011). The 11 loci were amplified twice for each sample, and the sample was removed if the 2 rounds yielded greater than 50% failure of the 22 total amplifications. I re-extracted and re-analyzed 6 randomly chosen samples that failed to amplify to determine if re-extraction would yield better results. To estimate the minimum number of markers needed for distinguishing between individuals, I calculated the probability of identity ($P_{(ID)unbiased}$; Paetkau et al. 1998), and the probability of identity among siblings with codominant loci ($P_{(ID)sib}$; Evett and Weir 1998) using Cervus 3.0 (Kalinowski et al. 2007). I accepted the minimum number of markers needed to achieve a $P_{(ID)sib}$ of < 0.001 for analyses (Waits et al. 2001). I removed 2 loci with the highest failed amplification rates, and individual consensus genotypes of the 9 loci were determined after independently amplifying loci. I followed the multiple tube approach recommended by Taberlet et al. (1996), and accepted 2 matching heterozygote and 3 matching homozygote alleles at each locus, where alleles were confirmed in replicate PCR reactions. Each round of PCR included an extraction negative of reagents to monitor contamination. I quantified genotyping errors from loci with consensus genotypes by calculating mean allelic dropout and

false allele rates (Broquet and Petit 2004). I retained a genotype for analysis if at least 5 of 9 loci were assigned a consensus allele score.

I identified sex of each sample by analyzing Y-chromosome deletions in the zinc-finger region (Pilgrim et al. 2005; Ruell et al. 2009). This method provides a positive for both sexes and minimizes interference with prey DNA because Y-chromosome deletions are absent in the prey species of wild felids (Pilgrim et al. 2005). I used PCR and the following primer sequences resulting in Y- and X-chromosome products for males 163 and 166 base-pairs long and 166 base-pairs for females (Pilgrim et al. 2005):

- Zn-finger F 5′-AAGTTTACACAACCACCTGG-3′
- Zn-finger R 5′-CACAGAATTTACACTTGTGCA-3′

I amplified the zinc-finger region in10-µL reactions containing 5 µL of PCR master mix (*Taq* DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers), 1.8 µL double-deionized H_2O, 0.2 µL BSA (20 mg/mL), 0.5 µL of each forward and reverse primer at 10 µM, and 2 µL DNA extract. The PCR profile consisted of an initial denaturation at 94 °C for 10 min, followed by 45 cycles at 94 °C for 30 sec, 56 °C for 90 sec, 72 °C for 1 min, followed by a final extension at 72 °C for 30 min. For quality control, the PCR contained 2 male bobcat blood positives, 2 female bobcat blood positives, 1 PCR negative, and 1 extract from a coyote scat.

Evaluation of Noninvasive Genetic Data for Population Monitoring

I found matching genotypes using the Excel Microsatellite Toolkit (Park 2001). I evaluated a plot of mismatching alleles before and after additional rounds of PCRs (Paetkau 2004). I accepted closely matching genotypes if they contained up to 2 mismatching alleles to avoid adding "ghost" individuals to the population because of errors associated with genotyping noninvasive sources of DNA (Creel et al. 2003). I recorded capture histories of each individual for the 10 sampling occasions after identifying matching genotypes. I determined abundance using the number of unique genotypes identified.

I estimated population size (*N*) using a genetic capture-recapture approach in the computer program CAPTURE (Otis et al. 1978). I assumed population closure within the 10-day sampling period, and I combined sexes for population size estimation because of small sample size (Ruell et al. 2009). I compared estimates between a null model and 2 heterogeneity estimators. The null model (M_0) uses a constant probability of capture, while models M_h-jackknife (Burnham and Overton 1979) and M_h-Chao (Chao 1988) incorporate individual heterogeneity in capture probability. I estimated crude population densities (*D*) by dividing the population size estimate (*N*) by the size of effective sampling area (Ruell et al. 2009).

To confirm ocelot and bobcat occupancy of my study area during collection periods, I compared abundance indices derived from a grid of infrared cameras (Fig. 3.2). The camera grid consisted of 26 stations with 2 cameras per station, placed near game trails, and spaced 1-km apart. The cameras were set for continuous detection with a 30-sec delay between images. I recorded the number of independent bobcat and ocelot detections during the scat collection periods, and determined minimum population size from individual identifications over a 3-month period.

Finally, I evaluated the feasibility of genetic monitoring using noninvasive sampling based on time, scale, and effort required to perform monitoring activities. I determined average daily search time, and average search time for a felid-positive detection. The scale of this study was demonstrated through the total transect distance and estimated effective sampling area. Effort was determined based on the number of observers, method of transportation, and other technical considerations.

RESULTS

Species Identification

I collected 172 carnivore scats and identified 89.5% to species (n = 154); 18 samples failed to amplify. I identified 0 ocelot, 1 badger *(Taxidea taxus),* 72 bobcat, and 81 coyote *(Canis latrans)* scat samples. No sequences of prey DNA amplified. I identified 4 variable sites with 3 haplotypes in the 20 bobcat sequences, and 1 haplotype in the 5 ocelot sequences, resulting in 15 variable sites with 89.7% identity between bobcats and ocelots (Fig. 3.5, Table 3.1). I identified 22 variable sites among bobcats, ocelots, jaguarundi, and mountain lion sequences (Fig. 3.4).

Individual Identification

Failed amplification rate of frozen bobcat samples (n = 33) using 11 loci was 66.9% (SD = 13.9%)(Table 3.2). Loci FCA035 and FCA096 were removed from analysis after failed amplification rates of 79.6% and 88.7%, respectively. Selecting absent or mismatching allele calls for additional rounds of PCR improved consensus matching; 2 pairs differed by 1 allele and no pairs differed by 2 (Fig. 3.6). With the remaining 9 loci, failed amplification rate of frozen bobcat samples was 63.3% (SD = 12.6%; Table 3.2). Of the frozen bobcat samples, 8 were female, 0 male, and 25 failed to amplify from the zinc-finger region. Since only 2 bobcat scats met genotyping requirements for frozen samples, samples collected during the 14 days in 2016 were removed from further analysis.

Failed amplification rate of DETs bobcat samples (n = 39) using 11 loci was 28.3% (SD = 9.9%; Table 3.2). Loci FCA035 and FCA096 were removed from analysis after failed amplification rates of 36.9% and 47.9%, respectively. With the remaining 9 loci, failed amplification rate of DETs bobcat samples was 22.6% (SD = 7.2%). Of the DETs bobcat samples, there were 22 female, 11 male, and 6 that failed to amplify from the zinc-finger region. Twenty-six bobcat scats met genotyping requirements for DETs samples and were included in analysis of individual identification and population size. The average consensus genotype was

8.23 of 9 loci (*SD* = 1.27). Of these, 20 individual bobcats were identified, including 12 females and 8 males (Fig. 3.7). Observed $P_{(ID)unbiased}$ was $7.2X10^{-10}$ and $P_{(ID)sib}$ was 0.00027 at 9 loci. There was no significant difference between original and re-extracted samples in amplification rates (*n* = 6, 71.9% vs. 72.2%). Mean allelic dropout rate was 13.8% (*SD* = 7.5%), and false allele rate was 2.8% (*SD* = 3.1%).

Population Size Estimation

The 2 female bobcats identified in 2016 were not included in population size estimates. I identified a minimum population size of 20 individual bobcats (12 female, 8 male) from 26 genotypes collected in the 10-day period in 2017. I assigned a match to 2 genotypes that differed by 1 mismatching allele after multiple rounds of replication amplified one of the consensus heterozygote alleles, likely indicating allelic dropout. The 6 other comparisons had exact matches. There were 4 female and 2 male recaptured bobcat samples. This consisted of 3 individual females (2 caught twice and 1 caught 3 times) and 1 male (caught 3 times). No individual bobcat was collected twice in the same sampling occasion.

Bobcat population size estimate with the null model (M_0) was *N* = 47 bobcats (SE = 16.8, 95% CI = 29-102), p-hat = 0.0532. Model M_h-jackknife estimated *N* = 79 (SE = 17.62, 95% CI = 53-124), average p-hat = 0.0316. Model M_h-Chao estimated *N* = 165 (SE = 161.05, 95% CI = 45-860), average probability of capture = 0.66. Effective sampling area was 111.1 km^2. Using only the minimum population size of the 20 unique bobcats, minimum bobcat density was estimated at 0.18 bobcat per km^2, or 5.56 km^2 per individual bobcat. The 3 occasions were impacted by rain were only able to produce consensus genotypes from 36% of bobcat scats compared to 79% of scats collected in fair weather. The rain impacted the 7th, 8th, and 9th sampling occasions, which likely contributed to the low frequency of recaptures.

Camera stations recorded 6 ocelot, 36 bobcat, 61 coyote, and 0 badger detections during the same collection periods. Therefore, ocelots were confirmed to occupy the study area during

scat sampling periods. Poor-quality images make camera individual detection difficult, but 3 months of camera trapping detected a minimum bobcat abundance of 12 individuals and a minimum ocelot abundance of 5 individuals. I identified twice as many bobcat scats as I did camera detections. Average transect sampling time was 3 hr 55 min per day with 1 observer and utility vehicle. The frequency of encounter for bobcat scat was 9.32 km per bobcat scat.

DISCUSSION

I was able to amplify quality mitochondrial DNA fragments from the ATP6 gene for almost 90% of the scats collected, comparable to previous studies (Ruell and Crooks 2007). The sequences targeted by the primers contained sufficient variation for distinguishing the wild felids that could be found in the area, without amplifying prey DNA. The ability to successfully identify species for carnivore scat from mitochondrial DNA amplification was not impacted by sample preservation protocol.

Results indicate that detecting the presence of bobcats and coyotes can be accomplished by collecting their scat, which can be found along ranch roads adjacent to their habitat. The most abundant source of carnivore scat on the roads was from coyotes, even after avoiding scats that were easily distinguishable as non-felid because of appearance and diet composition. The abundance of coyote scats easily detected along roads makes noninvasive genetic sampling an efficient option for coyote research (Kohn et al. 1999, Panasci et al. 2011). Badgers were rarely seen or detected on cameras in the study area, but an individual was detected with scat sampling methods.

Scat sampling along roads was an efficient method for detecting bobcats; 42% of the scats collected were identified as bobcat. However, the success of estimating bobcat population size and density was impacted by sample collection and preservation methods. Including both

years in the original robust design would have resulted in an unreasonably low capture rate due to failed amplifications for genotyping.

I was able to attain consensus genotypes from most bobcat scats collected in DETs, even though 3 mornings of rain lowered amplification success. The 3 model estimates have an imprecise 95% CI because of insufficient recaptured individuals. The minimum bobcat population size of 12 females and 8 males is most useful to report, and provides more detail compared to minimum population size estimates from 3 months of camera detections. The assumption of demographic closure was likely met during the short sampling period of 10 days, but geographic closure (movement on and off the study area) likely was not, as suitable habitat exists adjacent to the study area. Heterogeneity in individual capture probability is common in felid scat sampling (Ruell et al. 2009), which can cause population estimates to be biased low (Otis et al. 1978). Detection probability likely differs across sexes, social dominance, and how a home-range territory is delineated (Janecka et al. 2006, Ruell et al. 2009). For bobcats and ocelots, female home ranges rarely overlap those of non-related females, but male home ranges are larger and can overlap multiple female home ranges as well as those of other males (Bailey 1974, Tewes 1986, Lariviére and Walton 1997). Therefore, I expected to find more female than male scats, and the 1:2 (male: female) ratio of scats collected supports this prediction.

Using low-travel and unimproved ranch roads may not be a viable method of detecting the endangered ocelots in the U.S., even when camera data confirm their occupancy of a study area. I could have continued to add additional survey periods, but the cost of processing the high abundance of non-target samples and lack of ocelot detections suggests the method is inefficient. No studies of ocelots in Texas or Arizona have genetically confirmed an ocelot scat collected opportunistically. Opportunistic sampling along roads and trails for ocelot scat has been successful for their populations in Belize, where ocelots are more abundant (Wultsch et al. 2014), but the reasons for the lack of detections in this study are unclear. Ocelots visit home

range boundaries, mark their territory, and communicate with urine, similar to bobcat scent marking (Emmons 1988, Horne 1998). Also, ocelots likely defecate daily, as suggested by their rate of consumption of small prey (Emmons 1987). Ocelots also defecate at latrine sites for interspecific and intraspecific communication (King et al. 2017). A study in Panama found about 90% of ocelot scats in latrines with few found along trails (Moreno et al. 2006). Ocelots in South Texas may select latrine sites within habitat cover such as at the base of a tree or object (Murray and Gardner 1997) rather than defecating on roads. Bobcats and other felids will sometimes scrape the substrate around their scat, and may deliberately bury their scat (Elbroch 2003). However, this should not have been a factor in lack of detection because scrapes and disturbed soil made detection even more obvious in the moist sandy substrate.

MANAGEMENT IMPLICATIONS

Because trapping wild felids can be difficult, it is important to explore more efficient study methods. Laboratory costs are declining as technology progresses, and these expenses can be offset by reduced field costs using noninvasive collection of DNA (Schwartz et al. 2006). Noninvasive research has no negative impacts on the study animals. I suggest using the sampling methods in this study for application in bobcat research throughout their range. Future ocelot research should explore the use of scat-detecting dogs to aid in locating ocelot scat and latrine sites. It is easier to obtain permission from federal, state, and private landowners for collecting scat compared to trapping because the technique is minimally invasive and requires little disturbance when collection occurs on roads. This method can efficiently collect a large sample size with good coverage over a large area, even when the adjacent habitat is inaccessible. I recommend that sampling design for genetic capture-recapture should account for variable conditions such as rain and dung beetles affecting sampling occasions. I strongly recommend

using the DETs buffer in research that is using genetic information from carnivore scat in the South Texas environment.

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Figure 3.1. The East Foundation's El Sauz Ranch located on 110 km^2 in Willacy and Kenedy counties, Texas, USA, where scats were collected for bobcat and ocelot research during 2016 and 2017.

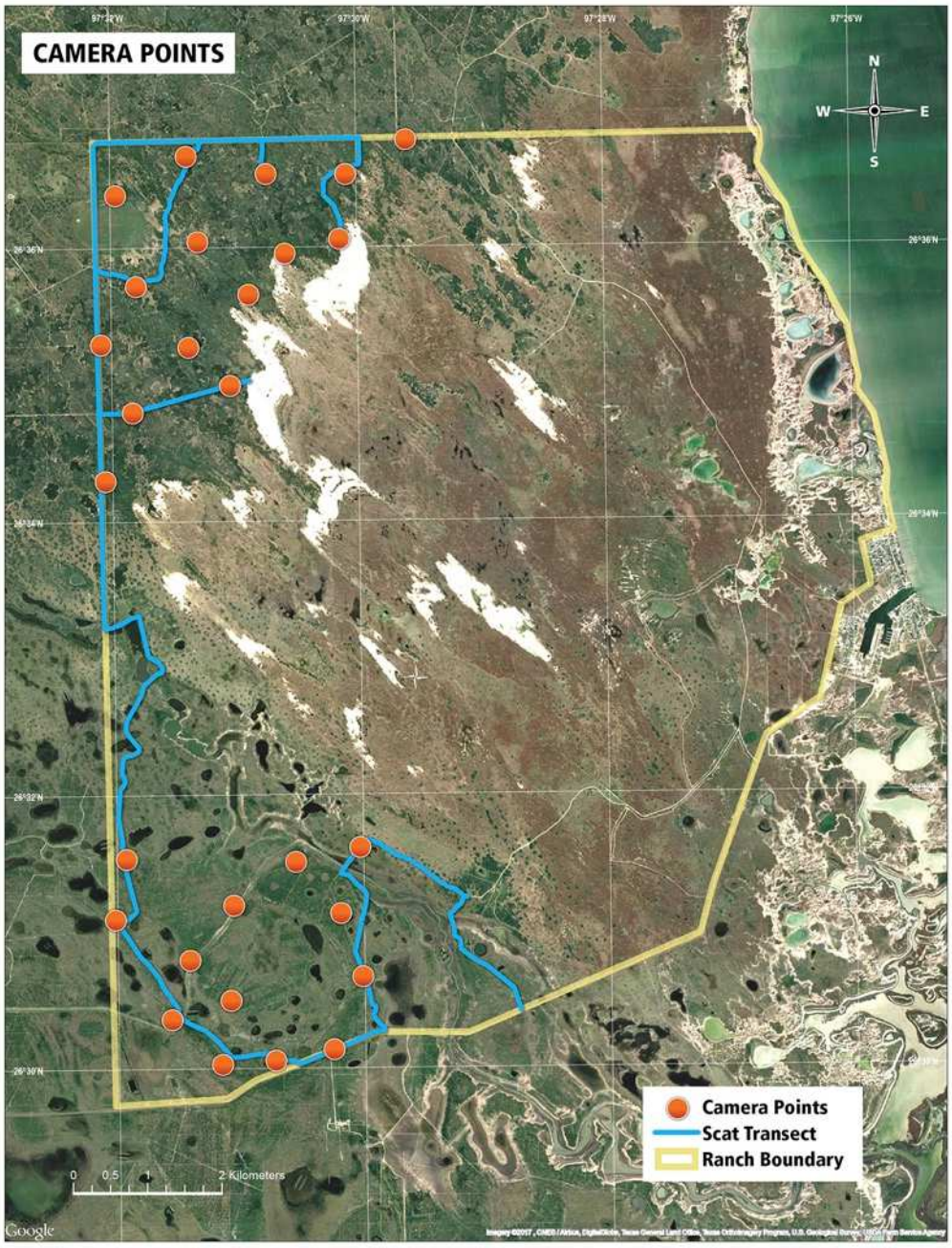

Figure 3.2. The East Foundation's El Sauz Ranch in Kenedy and Willacy counties, Texas, USA. Scat collection transects (34 km) were established in 2016 and 2017 on existing ranch roads in the northwest and southwest areas of the ranch, where ocelots and bobcats are known to occur. A camera grid, 26 stations with 2 cameras per station, was placed near game trails or roads and spaced about 1-km apart.

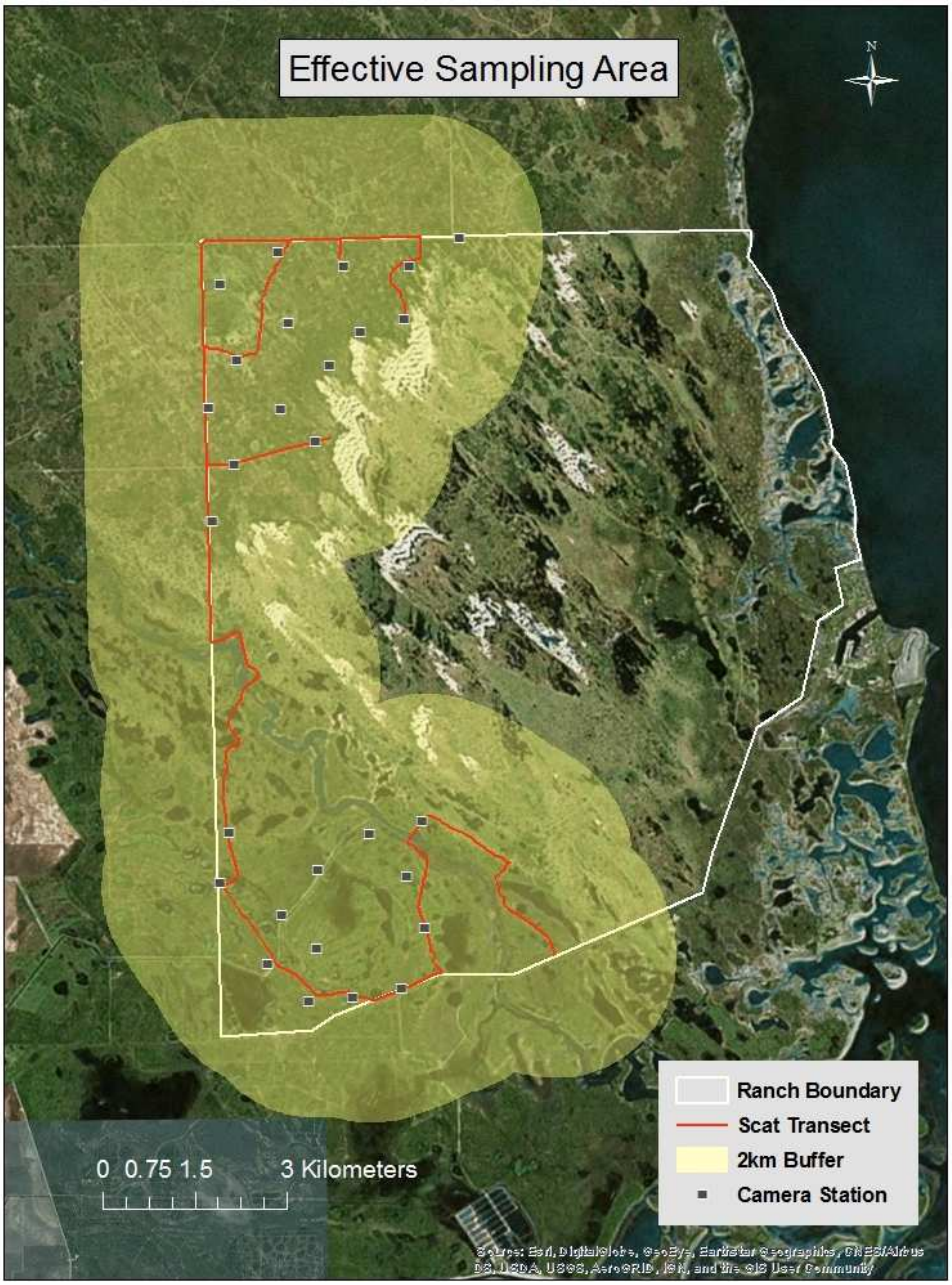

Figure 3.3. Bobcat and ocelot scat sampling transects used in a genetic capture-recapture study on the East Foundation's El Sauz Ranch in Kenedy and Willacy counties, Texas, USA, during 2016–2017. I estimated effective sampling area to be 111.1 km^2 based on a 2-km^2 buffer around 34 km of road transects. Two km represents the smallest ocelot home-range size reported for a female ocelot in South Texas (Navarro-Lopez 1985, Tewes 1986, Laack 1991). Sampling transects were established on ranch roads adjacent to known ocelot and bobcat habitat.

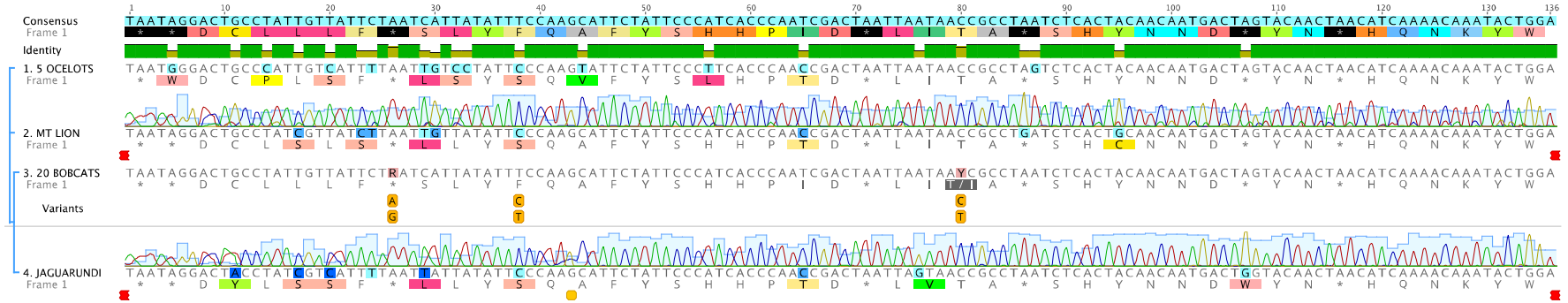

Figure 3.4. Sequences from the ATP6 mitochondrial gene of 20 bobcats, 5 ocelots, 1 mountain lion, and 1 jaguarundi aligned using program Geneious. I identified 22 variable sites between the 4 species. Bobcat and ocelot samples were collected from South Texas, USA, during 2012–2017, the mountain lion from West Texas, USA, 2010, and the jaguarundi from Tamaulipas, Mexico, 2004.

Table 3.1. A percent identity matrix constructed from mitochondrial DNA sequences of the ATP6 gene from 20 bobcats, 5 ocelots, 1 jaguarundi, and 1 mountain lion. Bobcat and ocelot samples were collected from South Texas, USA, 2012–2017, the mountain lion from West Texas, USA, 2010, and the jaguarundi from Tamaulipas, Mexico, 2004.

	20 Bobcats	5 Ocelots	Jaguarundi	Mt Lion
20 Bobcats		89.7%	92.6%	92.6%
5 Ocelots	89.7%		91.2%	91.2%
Jaguarundi	92.6%	91.2%		94.1%
Mt Lion	92.6%	91.2%	94.1%	

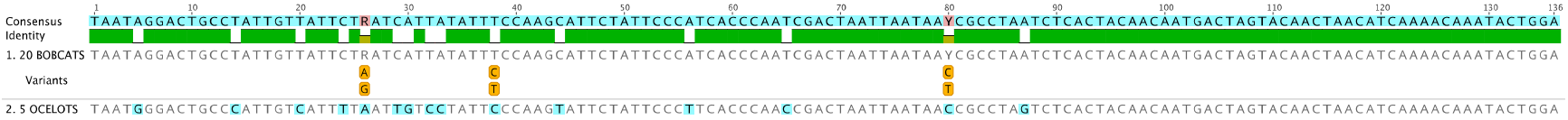

Figure 3.5. Mitochondrial DNA sequences from the ATP6 gene displayed 4 variable sites with 3 haplotypes in 20 bobcats, and 1 haplotype in 5 ocelot sequences, resulting in 15 variable sites. Genetic samples were collected from Kenedy and Willacy counties, Texas, USA, during 2012–2017.

Table 3.2. Genetic data from noninvasive sampling of bobcat scat in Kenedy and Willacy counties, Texas, USA, during 2016–2017 preserved frozen or in a DMSO/EDTA/Tris/salt (DETs) storage buffer. The DETs buffer performed better than frozen storage for preserving DNA for genetic analysis.

	Method	Bobcat Scats	Failed Amp. Rate	*SD*	Male Scats	Female Scats	Unknown Sex Scats	Consensus genotypes	*N*	No. Males	No. Females
2016 11 loci	Frozen	33	66.86%	13.91%	0	8	25	2	2	0	2
2016 9 loci			63.29%	12.58%							
2017 11 loci	DETs	39	28.27%	9.86%	11	22	6	26	20	8	12
2017 9 loci			22.60%	7.24%							

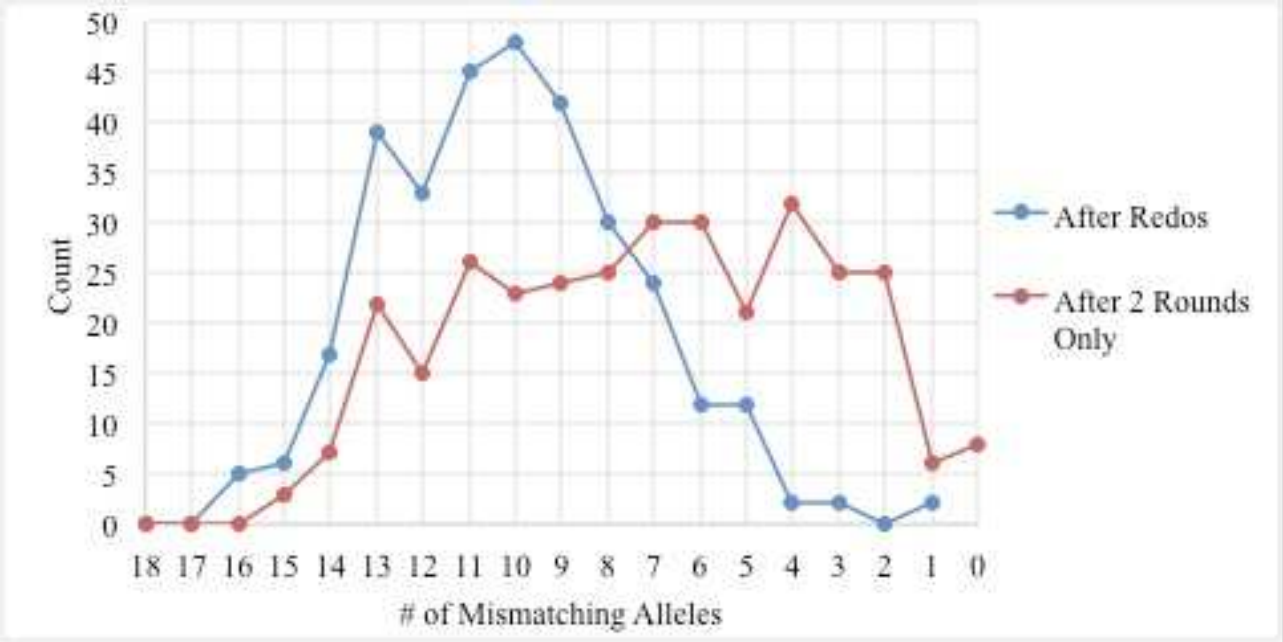

Figure 3.6. Mismatch distribution for 11-locus microsatellite DNA genotypes from noninvasive samples of bobcats collected in South Texas, USA, during 2016–2017. Plots depict frequency of mismatching alleles before and after verification using greater than 2 independent PCRs. I evaluated a plot of mismatching alleles before and after additional rounds of PCRs from consensus bobcat genotypes consisting of 11 microsatellite loci.

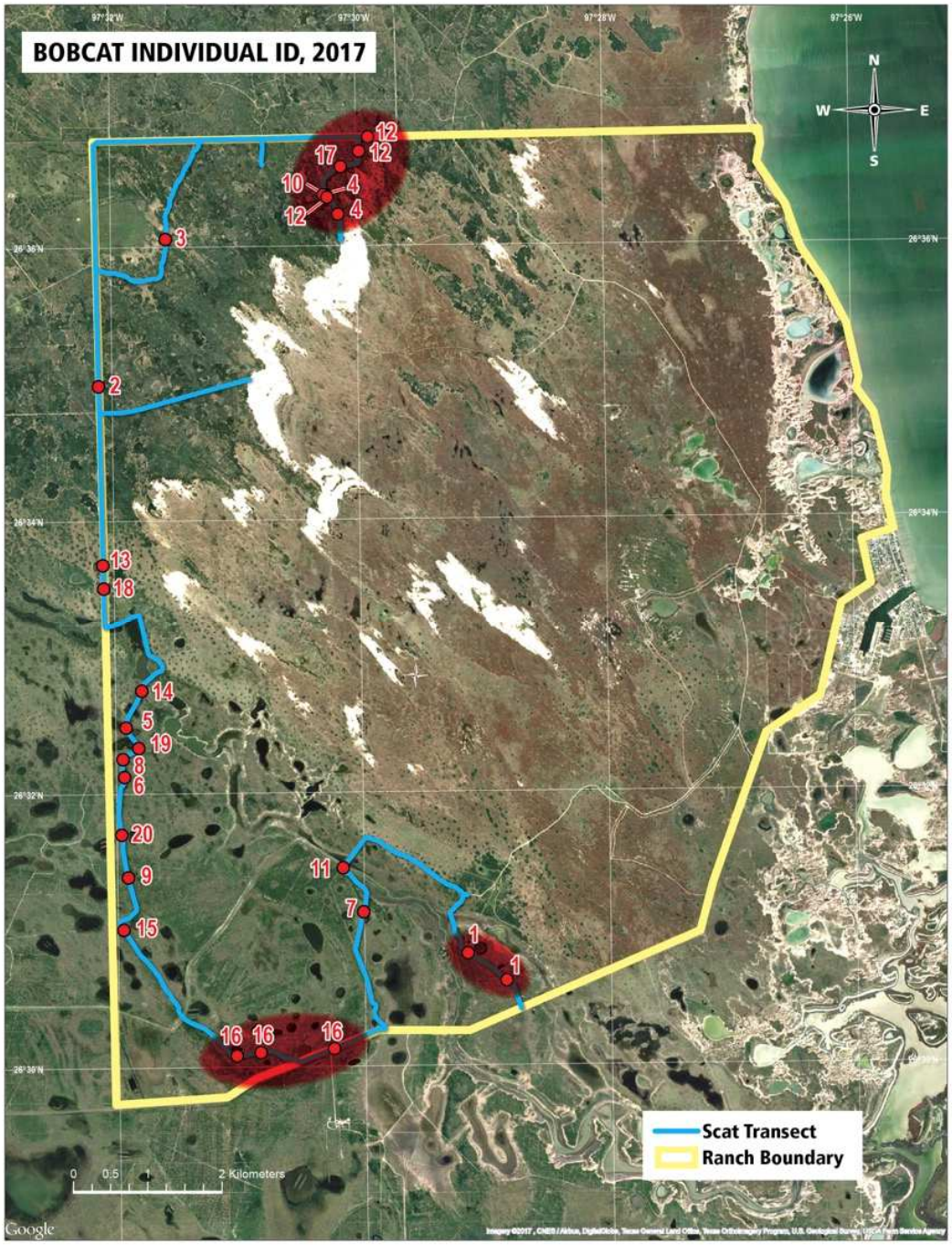

Figure 3.7. Distribution of individual bobcats identified by genetic capture-recapture analysis of scat samples. I identified 20 individual bobcats, identified by number, including 12 females and 8 males on the East Foundation's El Sauz Ranch in Kenedy and Willacy counties, Texas, USA, 2017.

CHAPTER IV

GENETIC ANALYSIS OF BOBCATS IN A FRAGMENTED LANDSCAPE

The maintenance of genetic diversity is essential for a population to avoid the deleterious effects of close inbreeding, limit susceptibility to disease, and adapt to environmental change (Frankham et al. 2002). Environmental and anthropogenic changes to wildlife habitat can impact how populations are connected across their range. Population genetics theory assumes that reproduction in an animal population without limitations would be random among all individuals, but this is seldom observed in nature (Reding et al. 2013). Limited connectivity of populations causes disruptions in gene flow and genetic structuring among habitat patches. This problem is especially the case in urban areas when the number of suitable habitat patches is insufficient or when corridors are ineffective. As habitat fragmentation threatens biodiversity, understanding the effects of anthropogenic landscape changes on natural populations is becoming increasingly important (Wilcove et al. 1998, Schwartz et al. 2006).

A suitable study location to evaluate the effectiveness of habitat refuges is among the rapid land-use changes in the Lower Rio Grande Valley (LRGV) of South Texas, where there are some of the highest rates of human population growth and development in the U.S. (Fulbright and Bryant 2002, Leslie 2016). Bobcat (*Lynx rufus*) populations are subject to these rapid landscape changes. Bobcats can be used as an indicator species for the effects of habitat fragmentation on wildlife populations because of their wide range, mobility, and adaptable generalist diet (Croteau et al. 2012). In contiguous habitats, studies of bobcat populations have revealed high connectivity and gene flow, where genetic differentiation across the species' range is a function of natural barriers (Millions and Swanson 2007) and topography (Reding 2011). However, contemporary research has shown anthropogenic factors, such as urbanization (Crooks 2002, Riley et al. 2006, Ruell et al. 2012) and agriculture (Reding 2011) fragment habitat and

Chapter IV of this thesis is written in the style of *The Journal of Wildlife Management.*

contribute to genetic differentiation among subpopulations of bobcats (Janecka et al. 2016). Therefore, habitat fragmentation that affects bobcats will likely affect other species of wildlife that are less adaptable in response to landscape changes (e.g., ocelot [*Leopardus pardalis*]). The overall goal of this study is to estimate the impact of fragmentation on genetic connectivity of bobcats in the borderland region of South Texas. The objectives of this research were to: (1) determine genetic diversity and population structure of bobcats in contiguous and fragmented habitats, and (2) determine the impact of fragmentation on genetic connectivity of bobcats in South Texas.

STUDY AREA

The study areas are located in South Texas within the Tamaulipan Biotic Provence (Blair 1950). This region has hot summers with daily high temperatures in the upper 30s (°C) and mild winters that frequently remain above freezing (Haines et al. 2006). Annual precipitation in the Lower Rio Grande Valley (LRGV) averages 64 cm, but is highly variable across years (Laack 1991). Woody habitat preferred by bobcats (Cain et al. 2003) is dominated by native thornshrub, honey mesquite *(Prosopis glandulosa),* and live oak *(Quercus virginiana)* communities. The predominant land use in the area consists of cattle and wildlife ranching, agriculture, energy development, and urbanization. The human population grew from approximately 400,000 in the 1960s to over 1.3 million in 2013 (Leslie 2016). The urban areas are mirrored south of the border in Mexico, where the city populations are more than 3 times higher (Leslie 2016).

I selected study areas with different degrees of surrounding fragmentation, from contiguous rangelands to urban areas (Fig. 4.1). The locations considered "rangeland" include the Rob and Bessie Welder Wildlife Refuge (WWF) in San Patricio county, the Santa Gertrudis Division of King Ranch (KRA) in Kleberg and Jim Wells counties (Korn 2013), East Foundation's Santa Rosa Ranch (SAR) in Kenedy County, Yturria Ranch (YTU) and East

Foundation's El Sauz Ranch (EES) in Kenedy and Willacy counties, Texas. The locations considered "fragmented" include The Nature Conservancy's Southmost Preserve (SOM) in Cameron County, and 7 "Las Palomas Wildlife Management Areas." These include the Arroyo Colorado unit (ACO) in Willacy and Cameron counties; Longoria (LON), Carricitos (CAR), Tucker-Deshazo (DEZ), Ebony (EBO), and Anacua (ANA) units in Cameron County; and Taormina unit (TAO) in Hidalgo County, Texas. These areas are managed for wildlife habitat among the urbanization of the LRGV and range from 410 ha on the SOM to as small as 47 ha on CAR. The SOM and ANA are adjacent to the Rio Grande and have land on both sides of the U.S.–Mexico border wall. These sections of the wall were built in 2006 consisting of 6-m-high steel posts with intermittent openings at various roads for vehicle access. There are 56 km of border wall in Cameron county and 35 km in Hidalgo county as of 2016 (Leslie 2016).

METHODS

Sample Collection

Roads and trails are commonly used in carnivore scat sampling (Kohn et al. 1999; Ruell et al. 2009, 2012) because of the higher probability of detection compared to randomized locations. Bobcats use roads and trails as regular movement corridors, and they frequently defecate along them for communication and territorial marking (MacDonald 1980; Kohn et al. 1999). I sampled each study area using roads and trails to find and collect scats that were visually identified as bobcat scat. I searched for scat by either traveling in a truck, utility vehicle, or on foot depending on the visibility of the substrate and accessibility. I recorded GPS location and used 2 principal methods of scat preservation, freezing and a buffer. During 2016, I collected whole scats by inverting a plastic bag or 532 mL whirl-pak (Nasco, Stamford, CT), and added 1-3 5-g absorbent packets of MiniPax® silica gel to remove moisture and prevent molding and degradation of the sample. Samples were kept in a shaded compartment in the vehicle and

transported to a cooler with dry ice within 4 hr from the time of collection. Samples were kept in the cooler with dry ice between 2-8 days before being transported to a -20 °C freezer. Samples were kept at -20 °C until immediately prior to extraction.

During 2017, I placed 0.4 mL of the outer surface of each scat into 2-mL screw top tubes containing 1.4 mL of DETs (DMSO/EDTA/Tris/salt) buffer (Frantzen et al. 1998). The DETs buffer consists of 20% dimethyl sulfoxide, 0.25M sodium-EDTA, 100 mM Tris, pH 7.5, and NaCl to saturation (Seutin et al. 1991). Samples were kept in a portable cooler while in the field and stored at 4 °C within 8 hr of collection. I wore disposable latex gloves and changed gloves between samples. I also used 9 DNA samples collected from the blood of trapped bobcats and extracted during a previous study in Kleberg and Jim Wells counties, Texas (Korn 2013).

DNA extraction

I extracted DNA in the Molecular Ecology Laboratory at Texas A&M University–Kingsville. I used the QIAamp® DNA Stool Mini Kit and the protocol recommended by the manufacturer (Qiagen, Hilden, Germany), with the exception that I performed the final elution step using 120 µL of storage buffer after incubation for 5 min. Each frozen sample was taken individually from the -20 °C storage and placed on a sterile disposable petri dish in the fume-hood. Forceps were cleaned with 95% ethanol prior to use for handling each sample. The surface of each frozen scat was shaved with a new razor blade to target epithelial cells remaining on the surface from the colon wall of the defecating animal (Reed et al. 2004, Rutledge et al. 2009). Enough scat was used to displace 400 µL of lysis buffer. For the DETs method, I began by homogenizing the samples in the 2 mL collection tubes of DETs buffer for 3 min using a vortex mixer. I pipetted 200 µL of homogenized scat into a tube of lysis buffer by cutting the end of a 1,000-µL filtered pipette tip to prevent clogging. I performed extractions in sets of 12, with 11 samples and 1 negative control, or in sets of 24, with 23 samples and 1 negative control. Extraction negatives contain only reagents but are treated as a sample during the extraction

process for quality control (Waits and Paetkau 2005). I stored DNA extracts at 4º C until further use. The blood samples were extracted using a DNeasy® Blood and Tissue Kit and the protocol recommended by the manufacturer (Qiagen Inc., Valencia, CA).

Species Identification

I sequenced a portion of the mitochondrial DNA ATP6 gene to verify species for each scat sample. I chose primers tested by Chaves et al. (2012) that distinguished between 66 species of carnivores, including bobcats, without amplifying non-carnivore species of prey. I confirmed the ability of the primers to distinguish bobcat from other species of felids that might be sympatric in the region, including ocelot, jaguarundi *(Puma yagouaroundi)*, and mountain lion *(Puma concolor)* using DNA extracted from blood or tissue during previous studies (Janecka et al. 2016, Holbrook et al. 2012, Holbrook et al. 2013). Each PCR included an extraction negative and known positive. The fragments were 172 base-pairs total, with 126 base-pairs excluding forward and reverse primers (Chaves et al. 2012).

- ATP6-DF3 (L7987) 5′–AACGAAAATCTATTCGCCTCT–3′ (Haag et al. 2009).
- ATP6-DR1 (H8114) 5′–CCAGTATTTGTTTTGATGTTAGTTG–3′(Trigo et al. 2008).

The 25-µL PCR for each sample contained 12.5 µL of AmpliTaq Gold® 360 PCR master mix (*Taq* DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers; Applied Biosystems, Foster City, CA), 8.3 µL double-deionized H_2O, 1 µL bovine serum albumin (BSA; 2 mg/mL; Thermo Fischer?Scientific, Waltham, MA), 0.1 µL of each forward and reverse primer at 100 µM (10 pmol), and 3 µL DNA extract. Thermocycling conditions were an initial denaturation at 94 °C for 3 min, followed by 10 cycles at 94 °C for 45 sec, a touchdown annealing temperature of 60 °C for 45 sec that decreased 1 °C each cycle to 50 °C, 72 °C for 1 min 30 sec. Next, 30 cycles of 94 °C for 45 sec, 50 °C for 45 sec, and 72 °C for 1 min 30 sec were followed by a final extension at 72 °C for 5 min. I chose to use 3 min initial denaturation instead of 10 min to allow the

enzyme activity from the hot start polymerase to increase over cycles as the amount of template increased (Lorenz 2012, Applied Biosystems).

I visualized PCR products with electrophoresis on a 1% agarose gel containing ethidium bromide and a 100 base-pair DNA ladder. Samples that did not show a band of the predicted size were removed from further analysis. I removed unincorporated primers and dNTPs from successful amplifications using an enzymatic method (ExoSAP-IT®, Affymetrix Inc., Santa Clara, CA). I cycle-sequenced the forward strands using the BigDye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems); dye terminators were removed using the DyeEx® 2.0 spin kit (Qiagen), and sequencing reactions were prepared for loading onto a 3130*xl* Genetic Analyzer (Applied Biosystems) following the manufacturer's recommendations. I edited and aligned sequences using the program Geneious® V10 (Kearse et al. 2012). I compared the sequences to references available in Genbank using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990). I determined species using a consensus of the top 3 results in NCBI-BLAST. Positive samples from trapped bobcat sequences were also used for reference. Sequences that aligned with non-target species or that failed to produce quality sequences were removed from further analysis.

Microsatellite Genotyping

Individual bobcats were identified using microsatellite genotyping. The empirically optimized protocol for a 10 µL PCR contained 5 µL of AmpliTaq Gold® PCR master mix, 1.8 µL double-deionized H_2O, 0.2 µL BSA (20 mg/µL), 0.5 µL of each forward and reverse primer at 10 µM, and 2 µL DNA extract. Thermocycling conditions included an initial denaturation at 94 °C for 10 min, followed by 10 cycles at 94 °C for 30 sec, a within-cycle decreasing annealing temperature of 62 °C for 30 sec, 61 °C for 30 sec, 60 °C for 30 sec, and an extension of 72 °C for 60 sec. Next, conditions for 40 cycles were 94 °C for 30 sec, 55 °C for 90 sec, and 72 °C for 60 sec followed by a final extension of 72 °C for 30 min.

I amplified 11 microsatellite loci in separate reactions, then combined reaction products into 3 panels for separation and detection. Loci included FCA008, FCA035, FCA043, FCA045, FCA077, FCA082, FCA090, FCA096, FCA132, FCA133, and FCA176 first isolated in the domestic cat (*Felis catus*) genome (Menotti-Raymond et al. 1999). These 11 loci were chosen based on polymorphism reported in previous studies of bobcats in Texas (Janecka et al. 2016; Davis 2015). Panel 1 was a mixture of 2 µL FCA008, 1 µL FCA043, 1 µL FCA082, 2 µL FCA090 and 5 µL double-deionized H_2O. Panel 2 was a mixture of 2 µL FCA035, 2 µL FCA096, 1 µL FCA132, 1 µL FCA133, and 5 µL double-deionized H_2O. Panel 3 was a mixture of 1 µL each FCA045, FCA077, FCA176 and 5 µL double-deionized H_2O. I denatured 1.1 µL of each diluted PCR product mixture with 0.5 µL GeneScan ROX500 size standard and 11 µL Hi-Di formamide (Applied Biosystems). The resulting mixtures were loaded on an Applied Biosystems 3130*xl* genetic analyzer for fragment separation and detection.

I scored alleles and merged into consensus genotypes using the computer program Genemapper® (Applied Biosystems). I calculated failed amplification rates as number of PCRs with the absence of a scored allele within the correct size range for each locus divided by the number of PCRs attempted at each locus (Panasci et al. 2011). The 11 loci were amplified twice for each sample, and the sample was removed if the 2 rounds yielded greater than 50% failure of the 22 total amplifications. I calculated the probability of identity ($P_{(ID)unbiased}$; Paetkau et al. 1998), and the probability of identity among siblings with codominant loci ($P_{(ID)sib}$; Evett and Weir 1998) using Cervus 3.0 (Kalinowski et al. 2007) to verify the ability to identify individuals genotypes. Individual consensus genotypes of the 11 loci were determined after independently amplifying loci. I screened for matching genotypes using the Excel Microsatellite Toolkit (Park 2001). I followed the multiple tube approach recommended by Taberlet et al. (1996), and accepted 2 matching heterozygote and 3 matching homozygote alleles at each locus, where alleles were confirmed in replicate PCRs. I accepted closely matching genotypes if they

contained up to 2 mismatching alleles due to errors associated with genotyping noninvasive sources of DNA (Creel et al. 2003). I retained a genotype for analysis if at least 5 of 11 loci were assigned a consensus allele score. Each round of PCR included an extraction negative of reagents to monitor contamination. I quantified genotyping errors from loci with consensus genotypes by calculating mean allelic dropout and false allele rates (Broquet and Petit 2004). Allelic dropout was calculated for each locus at heterozygous genotypes using the number of amplifications where 1 allele was lost divided by the number of positive heterozygous genotypes for that locus according to the consensus genotype (Taberlet et al. 1996; Broquet and Petit 2004). False allele rates were calculated at each locus by taking the number of amplifications where an additional allele was scored, and dividing that by the number of amplifications at that locus (Broquet and Petit 2004). I calculated genotyping failure and error rates separately for frozen and DETs preservation methods.

I identified sex of each sample by analyzing Y-chromosome deletions in the zinc-finger region (Pilgrim et al. 2005; Ruell et al. 2009). This method provides an internal positive for both sexes and minimizes interference with prey DNA because Y-chromosome deletions are absent in the prey species of bobcats (Pilgrim et al. 2005). I used PCR and the following primer sequences, resulting in Y- and X-chromosome products for males of 163 and 166 base-pairs and a single 166 base-pair product for females (Pilgrim et al. 2005):

- Zn-finger F 5′-AAGTTTACACAACCACCTGG-3′
- Zn-finger R 5′-CACAGAATTTACACTTGTGCA-3′

I amplified the zinc-finger region in 10-µL reactions containing 5 µL of Amplitaq Gold PCR master mix, 1.8 µL double-deionized H_2O, 0.2 µL BSA (20 mg/mL), 0.5 µL of each forward and reverse primer at 10 µM, and 2 µL DNA extract. The PCR profile consisted of an initial denaturation at 94 °C for 10 min, followed by 45 cycles at 94 °C for 30 sec, 56 °C for 90 sec, 72 °C for 1 min, followed by a final extension at 72 °C for 30 min. For quality control, the

PCR contained 2 male bobcat blood positives, 2 female bobcat blood positives, and 1 PCR negative.

Genetic Analysis

I classified samples as "rangeland" or "fragmented" based on their sampling location (Fig. 4.1). I calculated the minimum number of individual bobcats per km^2 in the fragmented habitat patches. I examined 5 measures of genetic diversity across loci within each of the 13 study areas, within rangeland and fragmented classifications, and globally. I calculated allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E) corrected for sample size (Nei 1978), and individual inbreeding coefficient (F_I) using program SPAGeDi (Hardy and Vekemans 2002). I tested for departures from Hardy-Weinberg equilibrium for each locus across all samples and tested for significant values of F_I with SPAGeDi. Significance of F_I was calculated after 1,000 randomizations of gene copies among individuals. I estimated the level of genetic difference between rangeland and fragmented populations by calculating pairwise F_{ST} (Weir and Cockerham 1984) averaged across loci using Arlequin 3.5 (Excoffier and Lischer 2010). I tested statistical significance with 16,000 permutations of individual locations in Arlequin.

I estimated the number of distinct genetic clusters (K), and identified the probability of population assignment for each individual using Bayesian clustering algorithms in program TESS (version 2.3, Chen et al. 2007). I first analyzed a model without admixture (Chen et al. 2007), which does not use any *a priori* assumptions of an individual's location as in Pritchard et al. (2000). I analyzed a model with admixture that estimates the number of parental populations (Durand et al. 2009). The admixture model uses an individual's location as a Bayesian prior so that neighboring individuals are more similar than individuals further away (Durand et al. 2009). Both models use a Markov Chain Monte Carlo (MCMC) sampling algorithm. For each model, I conducted 5 iterations at each $K = 2$ through 10, with 50,000 iterations and 10,000 burn-in

iterations. I chose the best model of *K* using the smallest Deviance Information Criterion (DIC) for each run (Chen et al. 2007). The DIC calculates model deviance penalized by an estimate of the effective number of parameters (Spiegelhalter et al. 2002). I analyzed the "hard clustering" geographical assignment output from TESS for the best model to visualize population assignments spatially.

I quantified the impact of fragmentation on fine-scale genetic structure at the individual level using spatial autocorrelation and a Bayesian estimate of migration among rangeland and fragmented samples. I used spatial autocorrelation to determine dependence of individual genotypes to their neighboring genotypes as a function of distance, and Bayesian assignment methods to identify the location and direction of gene flow patterns (Manel et al. 2003). First, I measured relatedness using Moran's *I* statistic (Hardy and Vekemans 1999), which calculates relationship coefficients using individual level pairwise comparisons of genetic relatedness and geographic distance with program SPAGeDi (Hardy and Vekemans 2002). I established neighbor distance classes to 1 km, 5 km, then each increasing by 5 km. Estimates of the pairwise relatedness means were jackknifed over loci and analyzed for statistical significance after 1,000 permutations of individual locations. I compared patterns of fine-scale autocorrelation between rangeland and fragmented populations and quantified the spatial extent of genetic patches, where Moran's *I* was statistically different from 0 (Storfer et al. 2016).

Next, I determined migration rates and direction of gene flow between fragmented and rangeland populations on a localized scale using a Bayesian assignment method in program BayesAss v.3.0 (Wilson and Rannala 2003). This localized scale included bobcats in the southernmost counties, including Kenedy, Willacy, Cameron, and Hidalgo (Fig. 4.2). I analyzed contemporary gene flow in a 2-population model between fragmented and rangeland populations, followed by a model that estimated migration among 10 sampling locations. Each analysis was run 5 times with different initial seeds for the random number generator to assess

consistency among runs. For each run, I performed 10 million iterations of a MCMC with an initial burn-in period of 1 million and a sampling frequency of 100. I adjusted the delta values of the migration rate, allele frequency, and inbreeding coefficient mixing parameters to achieve an appropriate acceptance rate to proposed changes between 20 and 60% (Wilson and Rannala 2003). These values were set at 0.3, 0.6, and 0.6, respectively. I analyzed model performance of the log-probability of the MCMC run with a trace file output visualized in program Tracer (Tracer Version 1.6, tree.bio.ed.ac.uk/software/tracer, accessed 31 October 2017). I selected each 2 population and 10 population run with the best model convergence and lowest deviance; delta *k* as calculated by -2 log Pr(X/*k*) (Pritchard et al, 2000, Faubet et al. 2007, Oyler-McCance et al. 2016). I calculated migration rates per generation as the proportion of individuals in 1 population derived from the other population (Wilson and Rannala 2003).

RESULTS

I collected 350 carnivore scats between December 2015 and May 2017. I identified 88.9% of scats to species (n = 311), including 200 bobcat samples. The other 150 samples were either coyote (*Canis latrans*) (n = 110), American badger (*Taxidea taxus*) (n = 1) or failed to amplify a quality sequence (n = 39).

I obtained 136 consensus genotypes and identified 68 individual bobcats, 59 from scat and 9 from blood, where individuals were sampled 1–9 times. The 68 individual bobcats included 29 males, 36 females, and 3 that failed to amplify. Observed $P_{(ID)unbiased}$ was $1.09*10^{-13}$ and $P_{(ID)sib}$ was $1.8*10^{-5}$ at 11 loci. The $P_{(ID)unbiased}$ was $1*10^{-5}$ and $P_{(ID)sib}$ was 0.0108 at the 5 loci with the fewest number of alleles, representing the most conservative estimate for probability of identity based on genotyping requirements. Of the 59 individuals identified through scat samples, the average consensus genotype was 9.55 of 11 loci (SD = 2.04). Failed amplification rate from frozen bobcat scats (n = 61) using 11 loci was 55.77% (SD = 13.7%), and

only 21% (n = 13) met genotyping requirements. Of the frozen scats that met consensus genotyping requirements, mean allelic dropout rate was 2.76% (SD = 3.5%), and false allele rate was 1.97% (SD = 2.4%). Failed amplification rate from DETs bobcat scats (n = 139) using 11 loci was 18.11% (SD = 6.87%), and 82% (n = 114) met genotyping requirements. Of the DETs scats that met consensus genotyping requirements, mean allelic dropout rate was 8.93% (SD = 4.61%), and false allele rate was 3.88% (SD = 2.9%).

Genetic Analysis

I identified 42 bobcats in rangeland and 26 in fragmented classifications. I identified 26 individuals from 85 genotyped scat samples collected in the fragmented areas, and identified 33 individuals from 44 genotyped scat samples collected in rangeland areas. Recaptured individuals accounted for about 70% compared to 25% in fragmented and rangeland areas, respectively. I found an average of 2.2 individual bobcats per km^2 in the fragmented habitat patches, ranging from 1.4 to 4.2 bobcats per km^2. Measures of genetic diversity, including A_R, H_O, H_E, and F_I, were not statistically different for rangeland, fragmented, and global multilocus averages (Table 4.1). Global H_O was 0.788 and ranged from 0.742 to 0.955 at 11 loci across study areas. I found a significant departure from Hardy-Weinberg equilibrium with a deficit of heterozygotes at locus FCA090 from the global population ($P \leq 0.001$). No other loci showed significant deviations (Table 4.2). Genetic differentiation (F_{ST}) between the 13 study areas was 0.04 (SE = 0.012) and 0.016 (SE = 0.004, P = 0.017) between rangeland and fragmented populations analyzed as 2 populations.

No distinct population subdivisions were identified in the Bayesian clustering models with or without admixture. The best models of K using DIC selection for without admixture and with admixture were K = 5 and K = 7, respectively. However, the model solutions appeared over-fit (Fig. 4.3) and displayed no evidence of geographic patterns of genetic structuring (Fig. 4.4).

I found 2 distinct patterns of spatial autocorrelation comparing rangeland and fragmented bobcats (Fig. 4.5). Moran's *I* values revealed positive spatial autocorrelation in the 1 km distance class for contiguous and fragmented areas. These mean pairwise relationship coefficients of 0.15 in contiguous and 0.21 in fragmented areas suggest averages of first cousin and half-sibling relationships within 1 km of an individual bobcat. No positive or negative patterns of spatial autocorrelation occurred beyond 1 km for bobcats in contiguous habitat, suggesting admixture throughout the population. However, I observed positive spatial autocorrelation up to about 25 km for bobcats within fragmented landscapes. Significant negative spatial autocorrelation occurred between 30 and 60 km, which indicates pairs of individuals were more genetically different at these distances than an average pair of individuals.

Comparing 2 populations, rangeland and fragmented, the log-probability plot for the lowest delta *k* MCMC run appears to have converged, with regular oscillations and no persistent high or low trends (Fig. 4.6; Wilson and Rannala 2003). Per generation, 71.4% (95% CI ± 7.8%) of individuals in the rangeland population derived from its own population, while 96.7% (95% CI ± 5.9%) of the fragmented population derived from the same population, suggesting restricted gene flow in fragmented areas. The direction of gene flow between populations was not equal, as 3.3% (95% CI ± 5.9%) of bobcats per generation in the fragmented population migrated from the rangeland, and 28.6% (95% CI ± 7.8%) of bobcats per generation in the rangeland population migrated from the fragmented areas. When the 10 study sites were analyzed as populations, directional gene flow was not statistically supported among fragmented sites, with the exception of SOM and LON (Table 4.3). Per generation, 11.7% (95% CI ± 8.6%) of individuals in LON (n = 4) derived from SOM (n = 6). Gene flow occurred between CAR (n = 2), EBO (n = 3), EES (n = 26), and YTU (n = 4) (Table 4.3; Fig. 4.2). The log-probability plot for the lowest delta *k* MCMC run that includes analysis of the 10 study areas had semi-irregular oscillations with some high and low trends (Fig. 4.7).

DISCUSSION

The goal of this study was to estimate the impact of fragmentation on genetic connectivity of wildlife in the South Texas borderland region using bobcats as an indicator species. I found evidence for restricted dispersal of bobcats occupying fragmented habitat patches in the LRGV. Analysis at the individual level detected significant spatial autocorrelation of genotypes, where restricted gene flow was evident in the fine-scale autocorrelation in fragmented habitats (Fig. 4.5) (Sokal et al. 1989, Peakall et al. 2003). No significant positive autocorrelation in rangeland bobcats beyond 1 km indicates relatively high dispersal and admixture, while the positive autocorrelation up to about 25 km for bobcats in fragmented areas indicates restricted dispersal (Epperson 2005). Significant negative autocorrelation at long distances for bobcats in fragmented areas indicates individuals at these distances are more genetically different than expected, suggesting isolation by distance. Moran's *I* coefficient is a close comparison to kinship coefficients (Epperson 2005). Thus, I observed values near first-cousin relationships (Moran's $I = 0.096$) even as far as 15 km for bobcats in the LRGV. It might not be possible for a bobcat dispersing for mating to travel this distance around urban areas to avoid mating with closely related individuals.

The Bayesian analyses of migration also indicate restricted gene flow, where migration between fragmented and rangeland populations was asymmetric. A dispersing bobcat born within the habitat patches of the LRGV is likely to remain within the fragmented areas or migrate to rangeland habitat if possible. However, the genetic evidence shows that bobcats in the rangeland are less likely to disperse to the fragmented areas. Inferences between specific study areas were limited because of small sample sizes. However, the small sample sizes are partly due to the size of the habitat patches; recaptured individuals accounted for about 70% compared to 25% in fragmented and rangeland areas, respectively. The high number of repeated individual encounters suggests that the fragmented habitat patches are not large enough to accommodate

new home range territories. Some of these habitat patches were occupied by multiple individuals despite the patch size being smaller than any bobcat home range in South Texas rangelands reported by Janecka et al. (2006). Considering the minimum number of bobcats in these habitat patches, I believe that their home ranges are comparable to those among the urbanization of coastal southern California reported by Ruell and Crooks (2007). The bobcat population occupying the LRGV could be a remnant source coping amongst the recent habitat changes rather than an area with immigration.

Bobcats have been able to tolerate landscape disturbances to some degree, and it was not uncommon for my research to observe bobcats occurring in urban areas (Hansen 2006, Riley et al. 2006). Overall, bobcats appear able to maintain genetic diversity, as genetic variability (H_O = 0.788) was similar to other studies of bobcats in South Texas (Ho = 0.742; Janecka et al. 2016). Satellite collar and observational data have shown bobcats use narrow corridors, such as drainage canals, for travel (Leonard 2017), which could provide access to the pockets of habitat. Additionally, farmlands may be used in dispersal during some times of the year (Kolowski and Woolf 2002).

I was not able to identify discrete genetic clusters of bobcats across the South Texas study locations. However, estimates of F_{ST} between study areas and between rangeland and fragmented populations suggest low levels of genetic structuring. Although bobcats may currently occupy these fragmented habitats, their long-term persistence in these environments is uncertain. Janecka et al. (2016) found more significant population structuring, with bobcat samples from the LRGV in a distinguishable cluster. Their study analyzed 8 of the same loci from bobcat samples collected about 1 to 2 decades prior to my study. The low levels of genetic differentiation in my research could suggest an increase in gene flow between bobcat populations within the past decade. However, the difference in results is also probably related to variation in sampling locations and sample size.

MANAGEMENT IMPLICATIONS

Based on the evidence for restricted dispersal of bobcats in the LRGV, it is likely that habitat specialists will be affected by fragmentation more severely. Additional research on restricted dispersal of bobcats in South Texas will facilitate large-scale conservation planning for other wildlife, including the endangered ocelot (*Leopardus pardalis*), a habitat specialist. Although all patterns of gene flow in bobcats are not indicative of similar responses in sympatric ocelots (Janecka et al. 2016), identifying sources of high movement resistances for bobcats will aid in conservation planning in areas of interest to future ocelot conservation. Highway mitigation projects are of particular interest in the coastal corridor of South Texas where the last 2 breeding populations of ocelots in the U.S. remain (Cain et al. 2003). An increased understanding of animal movement across these highways will aid in the decision-making for these road projects. Habitat patches that can be used as stepping-stones or travel corridors to more secure habitats should be protected or established to ensure sufficient dispersal from the bobcat population of the LRGV to favorable ranchland habitat. Restoration efforts should focus on creating habitat within 5 km of a source population.

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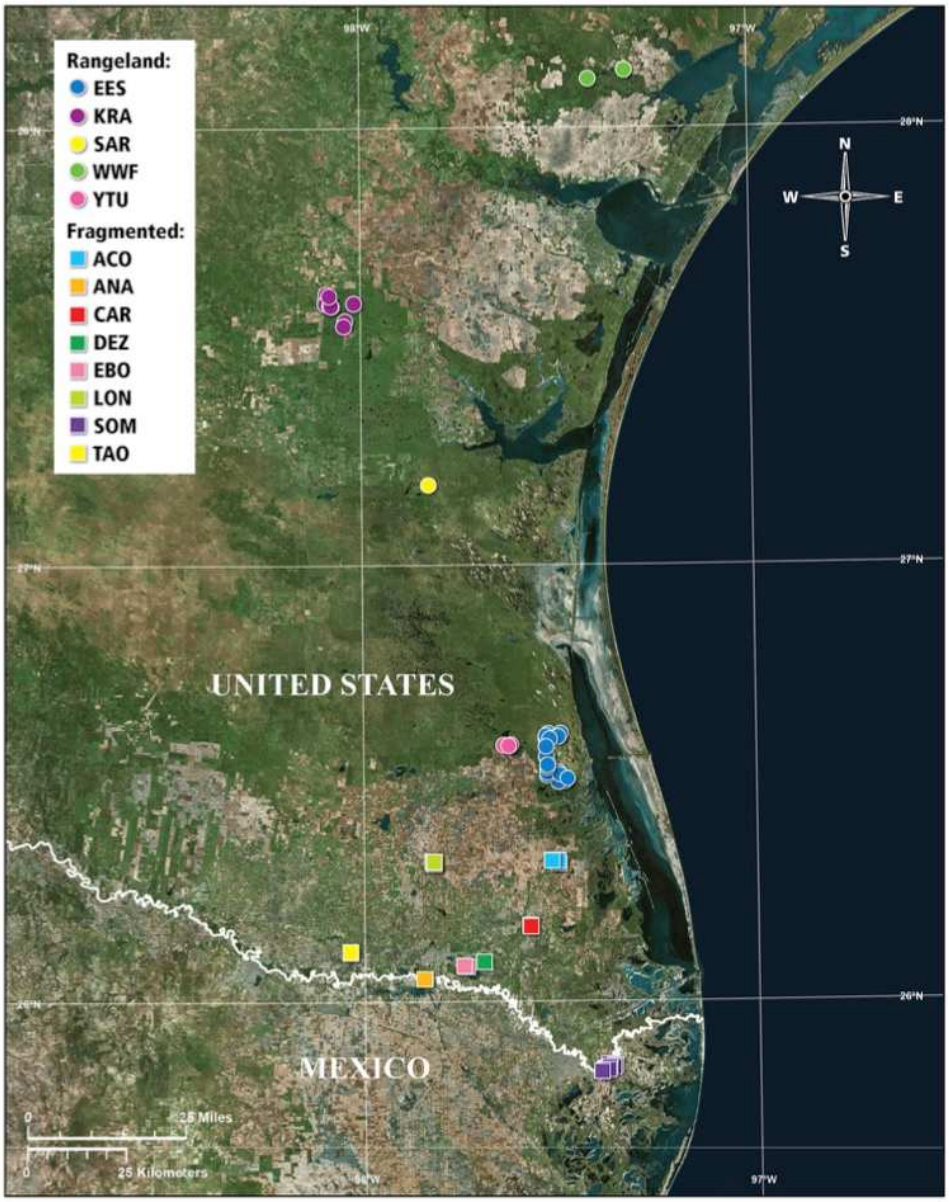

Figure 4.1. Locations of individual bobcats identified by microsatellite genotypes collected in study areas classified as fragmented or rangeland habitats in South Texas, USA, 2015–2017. Locations include the Welder Wildlife Refuge (WWF), Santa Gertrudis Division of King Ranch (KRA; Korn 2013), East Foundation's Santa Rosa Ranch (SAR) and El Sauz Ranch (EES), Yturria Ranch (YTU), The Nature Conservancy's Southmost Preserve (SOM), and 7 Las Palomas Wildlife Management Areas; Arroyo Colorado (ACO), Longoria (LON), Carricitos (CAR), Tucker-Deshazo (DEZ), Ebony (EBO), Anacua (ANA) and Taormina (TAO) units.

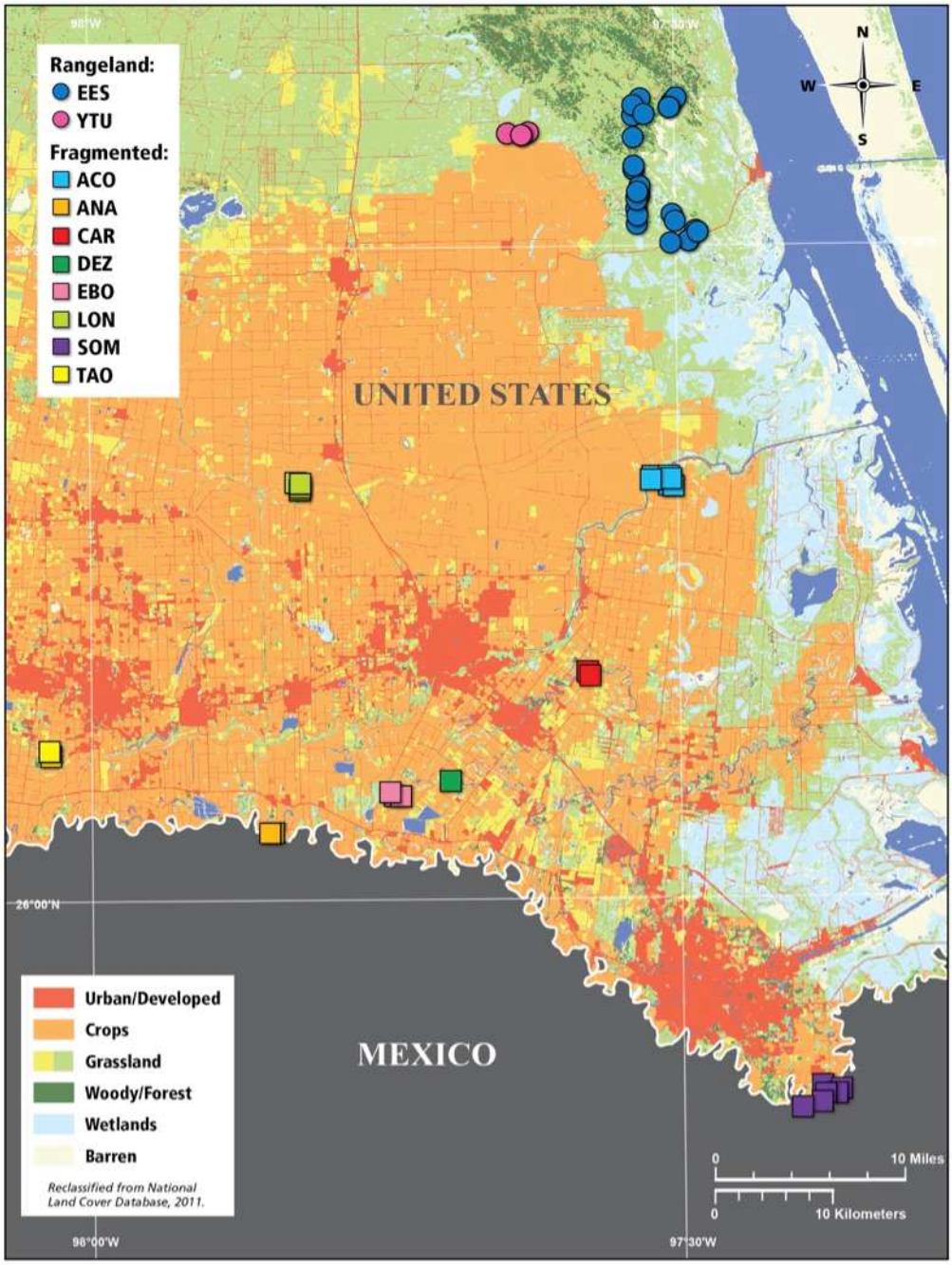

Figure 4.2. Locations of individual bobcats identified by microsatellite genotypes collected from study areas classified as fragmented or rangeland habitats in South Texas, USA, 2015–2017. I determined migration rates and contemporary gene flow in a 2-population model between fragmented and rangeland populations on a localized scale using a Bayesian assignment method of individual genotypes through program BayesAss v.3.0 (Wilson and Rannala 2003).

Table 4.1. Observed (HO) and expected heterozygosity (HE) corrected for sample size (Nei 1978), individual inbreeding coefficients (FI), and departures from Hardy-Weinberg equilibrium (HWE) for 11 microsatellite DNA loci amplified in bobcats sampled in South Texas, USA, during 2015–2017. Statistical significance was assessed using 1,000 randomizations of gene copies among individuals. *** = Deviated from Hardy-Weinberg equilibrium; NS = Not significant.

Locus	No. Alleles	*N*	H_O	H_E	F_I	*HWE*	*P*-value
FCA008	9	66	0.924	0.85	-0.088	NS	0.083
FCA043	6	67	0.731	0.757	0.034	NS	0.594
FCA082	8	61	0.869	0.806	-0.078	NS	0.169
FCA090	8	65	0.523	0.808	0.355	***	≤ 0.001
FCA035	15	50	0.9	0.875	-0.029	NS	0.607
FCA096	11	47	0.851	0.86	0.01	NS	0.856
FCA132	8	63	0.794	0.843	0.059	NS	0.303
FCA133	8	66	0.848	0.777	-0.092	NS	0.135
FCA045	7	64	0.734	0.76	0.034	NS	0.577
FCA077	9	60	0.733	0.733	0	NS	0.951
FCA176	6	63	0.762	0.712	-0.071	NS	0.309

Table 4.2. Genetic diversity of bobcats in South Texas USA, during 2015–2017 based on 11 microsatellite DNA loci. Study sites spanned fragmented landscapes and contiguous rangeland habitats. Allelic richness (AR) at K = 2, observed heterozygosity (HO), expected heterozygosity (HE) corrected for sample size (Nei 1978), and individual inbreeding coefficient (FI). Significance of FI calculated after 1,000 randomizations of gene copies among individuals

Location	*N*	No. Alleles	A_R	H_E	H_O	F_I	*P*-value (F_I<>0)
All Fragmented	26	7	1.77	0.773	0.793	-0.027	0.3646
ACO	6	4.45	1.72	0.715	0.742	-0.04	0.5624
ANA	2	2.73	1.74	0.742	0.773	-0.063	0.7463
CAR	2	3.09	1.85	0.849	0.955	-0.2	0.2128
DEZ	1	1.91	1.91	0.909	0.909		
EBO	3	3	1.69	0.694	0.803	-0.205	0.0679
LON	4	2.91	1.65	0.648	0.765	-0.217	0.0569
SOM	6	4.27	1.73	0.735	0.762	-0.041	0.6074
TAO	2	2.78	1.76	0.759	0.833	-0.204	0.7447
All Rangeland	42	7.91	1.81	0.805	0.787	0.023	0.2987
EES	27	7.18	1.8	0.797	0.76	0.047	0.0989
KRB	9	5.82	1.81	0.811	0.803	0.01	0.7932
SAR	1	1.82	1.82	0.818	0.818		
WWF	2	2.64	1.8	0.803	0.818	-0.037	0.8362
YTU	3	3.45	1.87	0.870	0.955	-0.163	0.1309
Global	68	8.64	1.8	0.799	0.788	0.013	0.4585

WWF = Welder Wildlife Refuge, KRA = Santa Gertrudis Division of King Ranch (Korn 2013), SAR = East Foundation's Santa Rosa Ranch, EES = East Foundation's El Sauz Ranch, YTU = Yturria Ranch, SOM = The Nature Conservancy's Southmost Preserve, and 7 Las Palomas Wildlife Management Areas; ACO = Arroyo Colorado, LON = Longoria, CAR = Carricitos, DEZ = Tucker-Deshazo, EBO = Ebony, ANA = Anacua and TAO = Taormina units

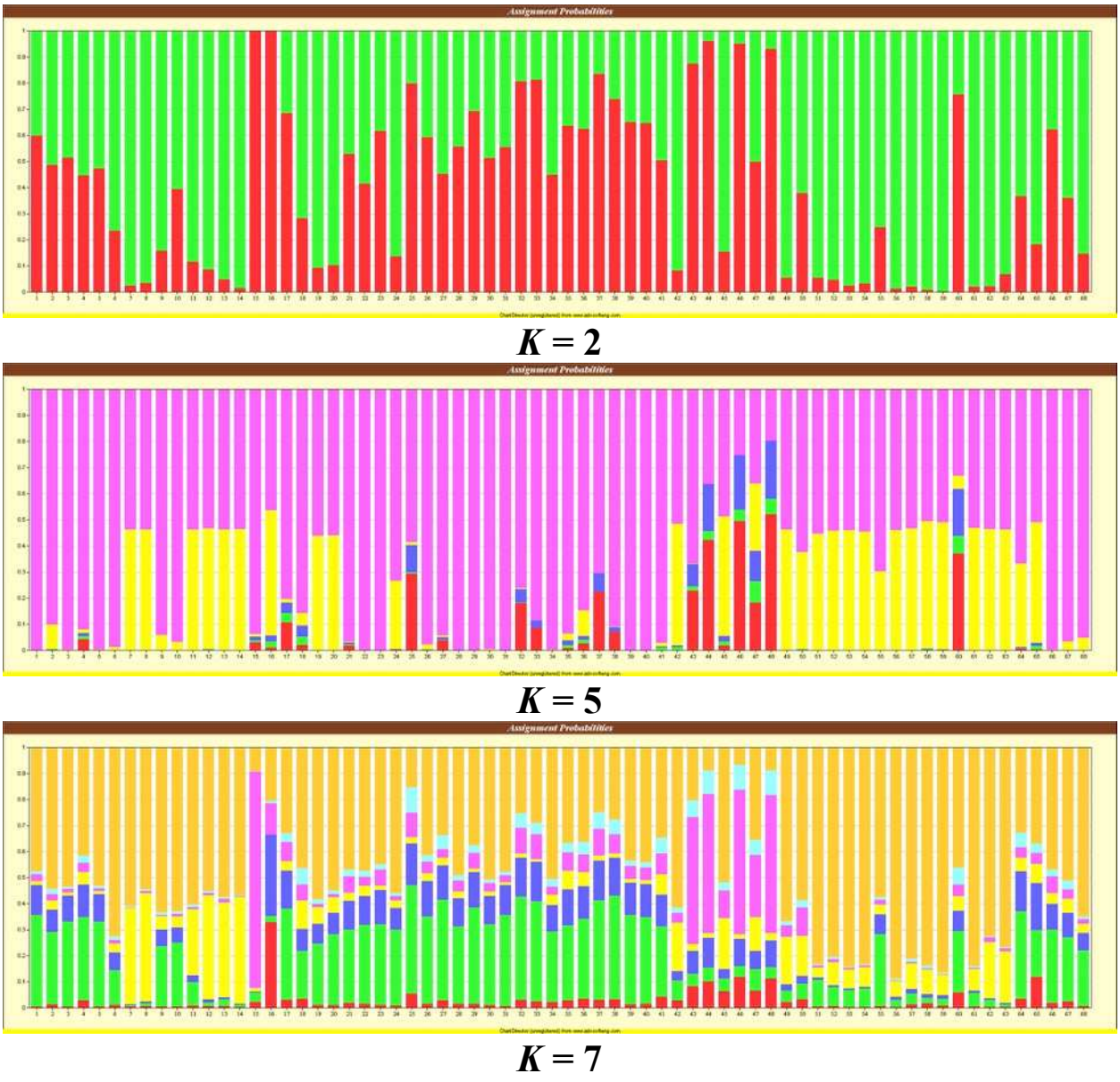

Figure 4.3. Likelihood of an individual, represented by a vertical bar, assigned to a cluster (*K*) with Bayesian models in program TESS (version 2.3, Chen et al. 2007) using microsatellite data from bobcats in South Texas USA, 2015–2017. The top performing models were K= 2 with admixture, K = 5 without admixture, and K = 7 with admixture. The admixture model uses an individual's location as a Bayesian prior. Models show no distinct clustering, indicating admixture and model over-fitting.

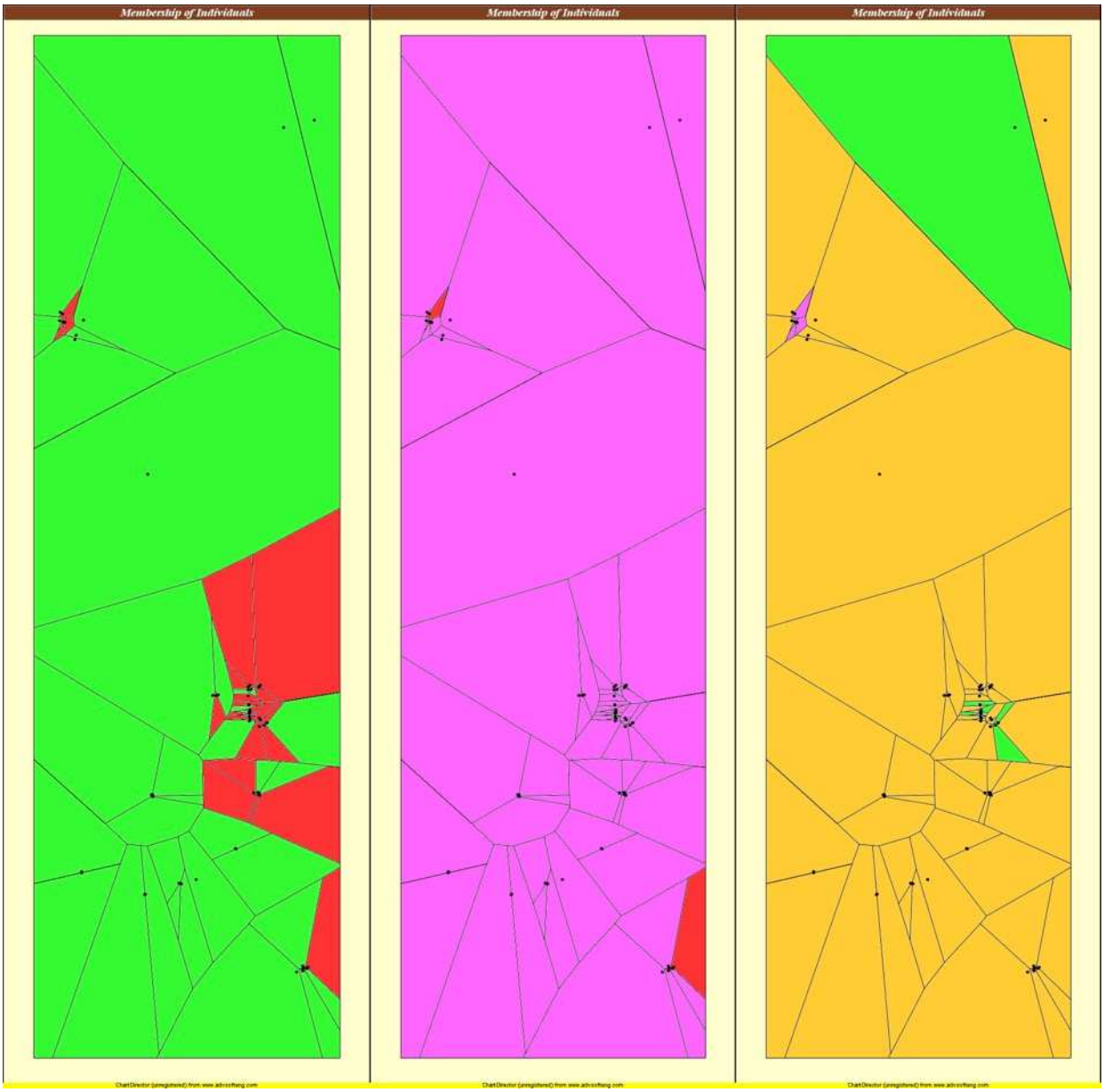

Figure 4.4. Geographic locations of individual bobcats in South Texas USA, 2015–2017 with likelihood of assignment to a distinct genetic cluster (*K*) using Bayesian models in program TESS (version 2.3, Chen et al. 2007). Using microsatellite data, the top performing models were K= 2 with admixture, K = 5 without admixture, and K = 7 with admixture. The admixture model uses an individual's location as a Bayesian prior. Models show no distinct population structuring with model over-fitting.

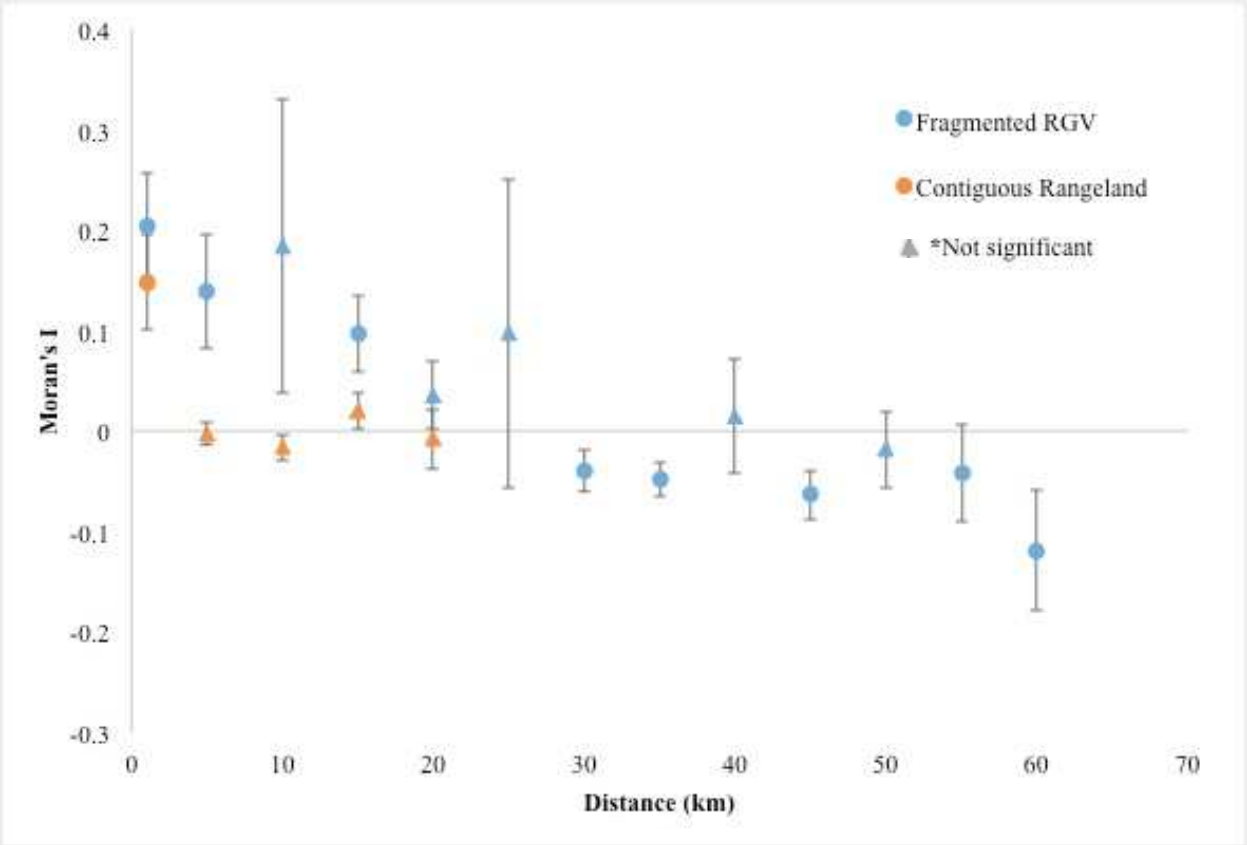

Figure 4.5. Spatial autocorrelation analyses of genetic data from bobcats in South Texas USA, 2015–2017 that occur within contiguous rangeland habitat or fragmented urban areas of the Lower Rio Grande Valley. Relatedness was measured using Moran's *I* statistic of pairwise comparisons and established distance classes. Bobcats in rangeland had genetic admixture beyond 1 km, and bobcats in fragmented areas show positive spatial autocorrelation up to about 25 km. Negative relatedness values at longer distance classes for bobcats in fragmented areas suggests restricted dispersal.

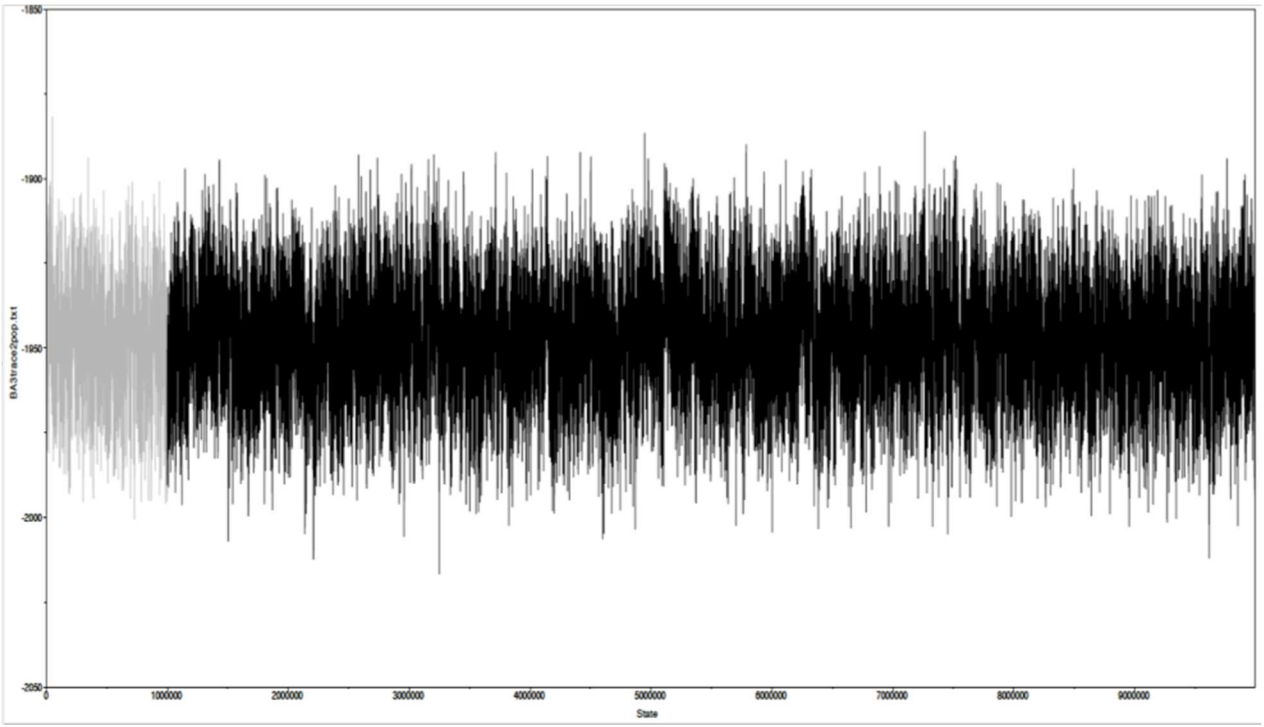

Figure 4.6. Log-probability plot of the Markov chain Monte Carlo analysis for recent migration from bobcat genotypes in program BayesAss v.3.0 (Wilson and Rannala 2003). Analysis used 10 million iterations and a 1 million initial burn-in period highlighted grey. The visualization from program Tracer appears to have converged, with regular oscillations and no persistent high or low trends (Tracer Version 1.6, tree.bio.ed.ac.uk/software/tracer, accessed 31 October 2017).

Table 4.3. Means of the posterior distributions of contemporary migration rate (m) and 95% credible intervals among bobcats sampled in South Texas, USA, 2015–2017. Migration rate was estimated using a Bayesian assignment algorithm based on data from 11 microsatellite DNA loci. Fragmented areas are Anacua, Arroyo Colorado, Carricitos, Ebony, Tucker-Deshazo, Longoria, and Southmost in Cameron county, and Taormina in Hidalgo county. Rangeland areas are El Sauz, and Yturria Ranch in Willacy and Kenedy counties. Mean values displayed are statistically different from 0. There was only one case of direction of gene flow between study areas within the fragmented areas.

	Migration from																											
	m	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI	*m*	LCI	UCI
Migration to		**ANA**			**ACO**			**CAR**			**EBO**			**DEZ**			**LON**			**SOM**			**TAO**			**EES**			**YTU**	
ANA	0.695	0.644	0.745																									
ACO				0.688	0.649	0.726																			0.132	0.047	0.217			
CAR							0.695	0.645	0.744																0.080	0.002	0.158			
EBO										0.693	0.645	0.740													0.099	0.018	0.180			
DEZ													0.697	0.643	0.751															
LON																0.692	0.642	0.743	0.117	0.031	0.203								
SOM																			0.752	0.664	0.840								
TAO																						0.695	0.644	0.746						
EES																									0.719	0.662	0.777	0.160	0.093	0.226
YTU																												0.693	0.645	0.740

EES = East Foundation's El Sauz Ranch, YTU = Yturria Ranch, SOM = The Nature Conservancy's Southmost Preserve, and 7 Las Palomas Wildlife Management Areas; ACO = Arroyo Colorado, LON = Longoria, CAR = Carricitos, DEZ = Tucker-Deshazo, EBO = Ebony, ANA = Anacua and TAO = Taormina units

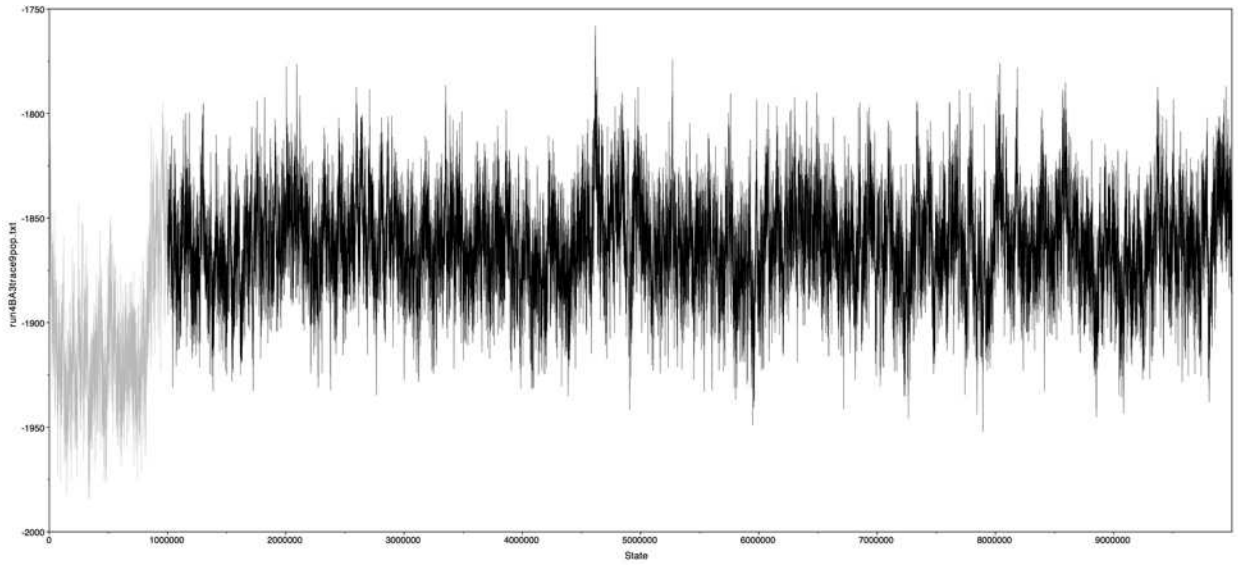

Figure 4.7. Log-probability plot of the Markov Chain Monte Carlo analysis for recent migration from bobcat genotypes of 10 populations in program BayesAss v.3.0 (Wilson and Rannala 2003). Analysis used 10 million iterations and a 1 million initial burn-in period highlighted grey. The visualization from program Tracer appears to have semi-irregular oscillations with some persistent low and high ends (Tracer Version 1.6, tree.bio.ed.ac.uk/software/tracer, accessed 31 October 2017).

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