GENETIC STRUCTURE OF THE NORTH AMERICAN PORCUPINE (*ERETHIZON DORSATUM*) ACROSS WESTERN TEXAS

by

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ABSTRACT

The North American porcupine (*Erethizon dorsatum*) is a highly mobile, generalist species with an extensive geographical distribution in North America. The porcupine was first documented in southwestern Texas in the early 20th century, but today occurs in most of the western two-thirds of the state. This species is relatively unstudied within the Great Plains ecoregion of North America, with no genetic studies having been conducted for this species in Texas. The objectives of this study were to describe population genetic metrics of porcupines across 3 ecoregions in western Texas by examining variation in 17 polymorphic microsatellites, and to confirm the applicability of the zinc finger protein sequencing method to identify sex in a population of North American porcupines.

Tissue samples from 106 porcupines were collected from the High Plains, Rolling Plains, and Edwards Plateau ecoregions of western Texas. Sex was accurately identified for 92 porcupine tissue samples by directly sequencing a short portion (195 base pairs) of the zinc finger protein gene. Sixteen base pair substitutions between Zfx and Zfy chromosomes denoted the sex of individuals; heterozygous sequence for males (Zfx and Zfy), homozygous sequence for females (Zfx only). All anatomically confirmed samples were correctly assigned to the known sex based on the generated sequence data: 51 male

and 41 female. Porcupines were genotyped for 17 polymorphic loci to estimate genetic variation and population structure. The variation in multilocus microsatellite genotypes for 100 porcupines support minimal genetic structure throughout the study area. Overall expected heterozygosity (H_E = 0.8327) exceeded observed heterozygosity (H_O = 0.7748). I observed moderate genetic variation with little population structure (K = 1). An overall F_{ST} = 0.0022 detected little to no divergence. STRUCTURE and Detrended Correspondence Analysis illustrated a primary genetic cluster with minimal grouping by ecoregion. An overall G_{ST} value of 0.0019 was obtained for porcupines across all ecoregions, suggesting that panmixia may be widespread throughout western Texas due to low variation of allele frequencies.

This research reveals that porcupines throughout western Texas are indeed vagile. The lack of population structure found in western Texas is likely the result of the relatively short life history and recent arrival of this highly mobile species within the state of Texas. The moderate genetic diversity reflects porcupine's wide use of habitat throughout the western portion of the state. This knowledge is beneficial in the management of this species, considered a pest by some, and to the overall understanding of the porcupine.

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"Dazzle them with brilliance and baffle them with bullshit."

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CHAPTER I

MICROSATELLITE ANALYSIS OF GENETIC VARIATION IN PORCUPINES ACROSS 3 ECOREGIONS IN WESTERN TEXAS

Introduction

The North American porcupine (*Erethizon dorsatum*) is the second largest rodent in North America and is most readily recognized by its quills (Roze and Ilse 2003). It is a generalist herbivore that exhibits major shifts in food habits between summer and winter months with a summer and spring diet consisting of grasses, forbs, and tree foliage, and a late fall and winter diet consisting almost exclusively of bark and cambium tissues of trees (Woods 1973, Sweitzer 1996). Long claws and a muscular tail make this species highly adapted for tree climbing. The porcupine is considered an arboreal species due to its year-round utilization of trees (Roze 1989).

The porcupine is well adapted to a variety of habitats including northern forests, tundra, rangeland, and deserts (Woods 1973), leading to an extensive geographical distribution that comprises most of the western, north-northwestern, and northeastern portions of the United States, parts of northeastern Mexico, and almost all of Canada and Alaska (Fig. I.1, Roze and Ilse 2003). The porcupine's wide-ranging diet and its ability to extract energy from nutrient-poor forage contribute to its widespread geographic distribution (Roze 1989, Felicetti et al. 2000). Typically a solitary, non-gregarious

mammal (Curtis and Kozicky 1944), its range has increased greatly across North America due to the reduction in natural predators, human distribution and land use, and habitat changes (Ilse and Hellgren 2001).

During the early 20th century, biologists in Arizona (Monson 1948, Reynolds 1957, Chew 1960), New Mexico (Ivey 1957), and Oklahoma (McMurry 1944, Tyler 1997) began noting a local porcupine population increase and range expansion from typical high mountain habitat down into lower mountain and plains habitats. Bailey (1905) was the first naturalist to document this species in southwestern Texas. By the mid-20th century, the porcupine could be found throughout in the southwest portion of the state with a southern range into the Davis Mountains, onto the Stockton Plateau, and as far east as Kerr and Mason counties (Taylor and Davis 1947). Milstead and Tinkle (1958) report observations suggesting northwest expansion into the southern Panhandle. The late-20th century has documented eastern expansion onto the Edwards Plateau (Ilse and Hellgren 2001,) and into north-central Texas (Dalquest and Horner 1984, Davis and Schmidly 1994). Today the porcupine occurs in most of the western two-thirds of the state with a range extending as far south as Hidalgo county and east to Bosque County with a recent county record in Van Zandt county (Fig. I.2). This range appears to be expanding farther still (Ilse and Hellgren 2001, Baird et al. 2009, Schmidly and Bradley 2016). However, this species is classified as endangered in northern Mexico (List et al. 1999). Three porcupine subspecies are recognized within the state of Texas: *E. d. epixantham* in the far western counties along the Texas and New Mexico border; *E. d. bruneri* in the northern Panhandle and some northeastern counties along the Texas and

Oklahoma border; and *E. d. couesi* throughout the remainder of the state (Fig. I.2, Schmidly and Bradley 2016).

Porcupines prefer habitats of mixed hardwood and softwood trees throughout their range. In open, non-forested areas, the porcupine is most often found inhabiting riparian areas, draws, and brushy stream bottoms (Woods 1973). Porcupine density is related to the distribution of habitat (Brander 1973, Smith 1977). It is probable that areas dense in those trees most favored as food could have local concentrations (Smith 1977). Porcupine population sizes vary between ecosystems and habitat types: 1.9 porcupines/km2 in pinyon-juniper woodlands of Texas (Ilse and Hellgren 2001);12.6 and 15.9 porcupines/km^2 in mixed pine-fir forest of Oregon (Smith 1977) and Nevada (Randall 1971) respectively; 37.2 porcupines/km^2 in mixed hard-wood hemlock forest of Michigan (Stoeckeler 1950); and a varying total population size of 82, 13, and 3 individuals across 3 subsequent survey periods of a 15km^2 enclosed basin within the Granite Basin Desert of Nevada (Sweitzer et al. 1997). The porcupine's only predators found in the southern Great Plains are the coyote (*Canis latrans*), bobcat (*Lynx rufus*), and the mountain lion (*Puma concolor*). Extensive predator control throughout the southern Great Plains and Texas has significantly reduced coyote and bobcat numbers. Mountain lions exist in the area, although in limited abundance (Schmidly and Bradley 2016). It is believed that where these predators are numerous porcupine densities may be lowered (Sweitzer et al. 1997).

Animal movements are activities associated with animal behavior and territory, including migration, immigration, and emigration. Both movement of individual animals and their incorporation into the breeding population are necessary for gene flow.

Genotyping of individuals can be used to assess this gene flow and determine paternity of individuals as well as the levels of population genetic structure. The extent of genetic differentiation, or variation, within a population affects its potential to adapt to environmental change (Frankham et al. 2007). Genetic differentiation among populations can help to reveal reproductive behavior, historical divergence of populations, and changes in ecological conditions due to human or natural forces (Bos et al. 2008).

Over the last 3 decades, conservation geneticists have developed a variety of genetic techniques for use by wildlife biologists to assess many genetic parameters within wildlife populations. Common types of molecular markers used include allozymes, mitochondrial DNA sequences, microsatellites, and minisatellites. These markers allow examination of taxonomic delineations, regional/subspecific population structure, genetic diversity, subpopulation structure, individual identification, and paternity/maternity analysis (Avise 1994, Frankham et al. 2002). Current population genetics studies use microsatellite markers to assess genetic variability within and between populations and apply those findings to knowledge of species ecology. These markers are selectively neutral and biparentally inherited by offspring, making them useful in population genetics studies (Bos et al. 2008).

Microsatellite markers are short tandem repeating segments of base pairs, usually 2-5 base pairs in length, located in the noncoding regions of nuclear DNA (Oyler-McCance and Leberg 2005, Pierce 2008). These markers allow a detailed description of a population's genetic structure by enumerating the types and frequencies of genotypes and alleles in a population. In conservation genetics, the primary focus of these

molecular markers is to tell wildlife biologists about the demography and genetics of a population and how that information can be applied to issues in wildlife conservation such as preserving the genetic diversity within and between populations of rare or endangered species.

Analysis of these markers has been used across an array of mammalian species to reveal support or refute hypotheses made about a population's genetic structure based on the animal's observed demographic characteristics and ecology. For example, monogamous mating and kinship of North American beavers (*Castor canadensis*) (Crawford et al. 2008) revealed multiple paternity in >50% of litters and extra-pair mating where multiple paternity is rarely reported in monogamous rodents; sex-specific dispersal pattern of wolverines (*Gulo gulo*) (Dalerum et al. 2007) supported high rates of dispersal among individuals but found no genetic patterns related to sex-biased dispersal as would be expected with male-biased dispersal behavior; and congruence and variation of microsatellite marker in the white-footed mouse (*Peromyscus leucopus*) (Schmidt 1999) found that said markers could correctly assign 92% of individuals to the correct cytotype, 63% to populations within a transect, and 100% within a study area to their respective populations.

Microsatellite primers are specific to a single locus and are usually specific to a particular species or group of closely related species (Frankham et al. 2002). As the North American porcupine is the only hystricomorph rodent in the United States and Canada (Wood 1950), no microsatellite loci were available from a closely related species until Barthelmess et al. (2013) developed 19 polymorphic microsatellite markers specifically for use in population genetic studies of the North American porcupine. Very

few other genetic studies have been conducted on the North American porcupine: Makino (1953*a*, *b*) noted a diploid chromosome number of 34 and the large size of the X chromosome, and Benirschke (1968) reported an unusual diploid chromosome number of 42, a large Y chromosome, and an X chromosome that is nearly twice the usual size of rodents. Genetic studies on other New World porcupine species include the use of various molecular markers to assess variability within and among populations of the thin-spined porcupine (*Chaetomys subspinosus*) (Oliveira et al. 2011), and molecular gender identification techniques for the prehensile tailed porcupine (*Coendou prehensilis*) (Woc-Colburn et al. 2013).

Movements of some populations of North American porcupines have been studied in relation to dispersal, mating, and resource selection, but porcupines in the Great Plains region of North America are relatively unstudied. This ecosystem varies greatly from others where extensive research has been conducted on this species. Studies within the Great Plains were conducted in Colorado (McLean et al. 1993, Synder and Linhart 1997), Montana (Hendricks and Allard 1988, Mally 2008) and Alberta, Canada (Harder 1980). These studies described winter food habits, hierarchical summer habitat selection, feeding patterns, and the ecology of porcupines in relation to Colorado tick fever. The literature reveals 3 studies in the state of Texas: Ilse and Hellgren (2001, 2007) described demographic, behavioral, and feeding characteristics of porcupines in pinyon-juniper woodlands of Texas, and D. Montgomery (West Texas A&M University, unpublished data) described the natural history and ecology of porcupines in the Texas panhandle. A. Montalvo (Texas A&M University College Station, personal communication) is presently

investigating cave use by porcupines in northern Bexar County, Texas. To my knowledge, there have been no genetic studies on this species in the state of Texas.

Identifying porcupine genetic structure will provide insight into relationships between separated populations of porcupines and an estimate of gene flow within and between potential populations. Information specific to the Great Plains geographic area will aid in management and conservation decisions, as well as the overall understanding of this species in Texas. The objective of my study was to describe population genetic metrics of porcupines across 3 ecoregions in western Texas by examining variation in 17 polymorphic microsatellites. Despite the mobility of the porcupine, dramatic landscape changes occurring across the study area led me to hypothesize that porcupines would be separated into 3 distinct populations based on locality across the 3 ecoregions, and because the porcupine is a highly mobile species, I hypothesized that low levels of genetic variation would be observed both within and between assumed populations.

METHODS

Study Area

One hundred and six porcupine tissue samples were opportunistically collected from September 2013 to December 2016 throughout portions of the High Plains, Rolling Plains, and Edwards Plateau ecoregions of western Texas (Fig. I.3).

The High Plains ecoregion is separated from the Rolling Plains ecoregion by the Caprock Escarpment in the east and dissected by the Canadian River Breaks in the north. Notable canyons include the Tule and Palo Duro. This high plateau is characterized by mesas and grasslands with a mixed prairie vegetation largely devoid of trees and brush.

Adjacent to the High Plains, the Rolling Plains ecoregion is comprised of gently rolling rangelands with steep slopes and canyons occurring just below the Caprock Escarpment. Heavy grazing and fire suppression have driven mixed prairie vegetation to a primarily mesquite-shortgrass savannah with stream floodplains being dominated by various hardwood species. Together, the High Plains and Rolling Plains form the Texas Panhandle. At the southern border of the Rolling Plains, the Edwards Plateau ecoregion is characterized by a flat elevated plateau with interfingering canyons in the west and a deeply eroded Hill Country in the east. Originally grassland savannah, this west-central Texas region is now predominately brushland with various poor-quality browse, forb, and grass species. These 3 ecoregions comprise the southernmost unit of the Great Plains within the central United States (Chambers 1946, Huser 2000, Shaw and Montgomery 2011).

DNA Sample Collection

Porcupines were captured using 2 methods: Tomahawk wire live traps (106.7 cm X 30.5 cm X 30.5; Tomahawk Live Trap, Tomahawk, WI) were baited with apples and salt and placed at the ends of culverts and the bases of occupied trees (Griesemer et al. 1999), and porcupines found on the ground were captured by hand using Kevlar-lined leather gloves (Shadle 1950). Approximate weight was recorded in kilograms and measurements in centimeters were taken for total body, tail, and hind foot length (Griesemer et al. 1999). Whenever possible, sex, approximate age, and reproductive status was recorded. GPS coordinates were recorded for each individual (O'Neil et al. 2005). Anatomical observations were recorded through photographic evidence and/or the 'Apple' application Theodolite (Hunter Research and Technology L.L.C.; accessible at

hunter.pairsite.com/theodolite). All capture methods and handling procedures met the requirements of the Guidelines for the Capture, Handling, and Care of Mammals as approved by The American Society of Mammalogists and West Texas A&M University Standard Operating Procedures SOP No. 15.99.05.W1.02AR Institutional Animal Care and Use (IACUC# 3-10-13).

For live-trapped individuals (N = 17), tissue samples collected for DNA analysis included quills with attached root follicles and fecal material. Animals were released at the site of capture. For individuals of vehicular casualties (aka. roadkill) (N = 89), postmortem tissue samples included hairs and quills with attached root follicles and muscle tissue. Hair, quill, and muscle tissues were stored either dry and frozen at -20 °C or submerged in 99% ethanol at room temperature. Fecal samples were stored frozen at -20 °C.

Microsatellite Genotyping

Genomic DNA extractions were performed on hair, quill, and muscle tissues using a modified DNeasy® Blood and Tissue Kit (Spin-Column Protocol) (QIAGEN Inc., Valencia, CA, USA) (Woc-Colburn et al. 2013). Genomic DNA was extracted from fecal material using the FastDNA® Sample Spin Kit Feces (MP Biomedicals, LLC, Solon, OH.) following the manufacturer's instructions.

Five mm of root tip was cut from 10-15 quills, 20-30 hairs and/or a pea-sized piece of muscle tissue was manually homogenized before being placed in a 1.5 mL microcentrifuge tube with 197 µL Buffer ATL and 3.0 µL of 25 mg/mL proteinase K (Research Products International Corp., Mt. Prospect, IL, USA). Samples were incubated

in a water bath 4-6 hours at 56 °C to ensure complete lysing and maximize yields. The extractions were completed on the second day by adding 200 µL of Buffer AL and 200 µL ethanol (99%) to each tube and vortexing thoroughly. Sample supernatants were then pipetted into a DNeasy® Mini spin column and centrifuged at 8000 rpm for 1 minute. Five hundred µL of Buffer AW1 was added to each column and samples were again centrifuged at 8000 rpm for 1 minute. Next, 500 µL of Buffer AW2 was added to each column and samples were centrifuged for 13,400 rpm for 4 minutes. Centrifuge rpm and speed was modified from the protocol's recommended 14,000 rpm for 3 minutes due to limited rpm capabilities of available centrifuges. Each DNeasy® Mini spin column was then placed in a new 1.5 mL microcentrifuge tube. Buffer AE was added to each column, incubated at room temperature for 1 minute, and centrifuged a final time at 8000 rpm for 1 minute. The amount of elution buffer (Buffer AE) was modified from the protocol's recommended 200 µL per sample, based on the detected concentration of DNA after an initial elution of 50 µL. DNA with low concentrations (<10 ng/µL) were kept at an elution volume of 50 µL and DNA with higher concentrations (>10 ng/µL) were eluted to volumes of 50-200 µL as needed. DNA concentrations were detected using a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a Qubit™ dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Genomic DNA samples were either maintained at 2.7 °C for immediate use or stored at -20 °C for future use.

Seventeen polymorphic microsatellite markers, developed and described by Barthelmess et al. 2013 (Table I.1), were used to successfully genotype 100 porcupines. Using O'Bryhim et al. (2012) as a guide, all polymerase chain reaction (PCR)

amplifications were performed in a 12.5µL volume with final reagent concentration and volumes as follows: 6.25µL of GeneMate Red Taq 2x Master Mix (BioExpress, Kaysville, UT, USA), 0.5 µL of 50.0 mg/mL bovine serum albumin (BSA), 0.5µL of 5µM Well-Red fluorescently-labeled D4-PA primer (Sigma-Genosys, The Woodlands, Texas, USA) (Table I.1), 0.5µL of 10µM tag labeled forward primer (Sigma-Aldrich Inc., St. Louis, MO, USA) , 0.5µL of 10µM unlabeled reverse primer (Sigma-Aldrich Inc., St. Louis, MO, USA), 20ng template DNA, and ddH_2O. The forward primer from each pair was modified on the 5' end with an engineered sequence (D4-PA tag 5'-GAGTTTCCCAGTCAC-3') to enable use of a third primer in the PCR (identical to the D4-PA tag) that was fluorescently labeled. A touchdown cycling program (Don et al. 1991) encompassing a 10 °C span of annealing temperatures ranging between 65-55 °C (TD65) was used for all PCR amplifications. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95 °C followed by 20 cycles of 95 °C for 30 s, highest annealing temperature (decreased 0.5 °C per cycle) for 30 s, and 72 °C for 30 s; and 20 cycles of 95 °C for 30 s, lowest annealing temperature for 30 s, and 72 °C for 30 s. All amplifications were performed on an Eppendorf Mastercycler® Gradient 5333 (Eppendorf, Hamburg, Germany). PCR amplicons were run for 45 min at 100 V in a 1% agarose gel stained with 10mg/mL ethidium bromide and visualized under UV light to determine successful amplification. Negative controls (no sample material added to the extraction) accompanied each set of PCR amplicons to check for possible contamination. PCR amplicons were then fractionated through capillary electrophoresis on a Beckman Coulter CEQ8000 DNA Analyzer (Beckman Coulter, Indianapolis, IN, USA). Microsatellite sizes identified by the CEQ8000 software were confirmed by visual

inspection of tracings. GeneMate 400 bp Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) was included on each analysis for each reaction as a size standard.

Microsatellite Data Analysis

Measures of genetic variability, such as alleles per locus (A_N), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphic information content (PIC) values for each locus were determined with Microsatellite Toolkit for Microsoft Excel (Park 2001). Tests for Hardy Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were performed using GENEPOP 4.2 (Raymond and Rousset 1995, Rousset 2008). Allele frequency-based correlations were examined by deriving population subdivision (F_{ST}) and inbreeding coefficients (F_{IS}) for each locus using GENEPOP 4.2. I tested for deviations from equilibrium within ecoregions at each locus and across all loci. The Bonferroni method was used to correct *p*-values for multiple comparisons in the HWE and LD tests (Rice 1989).

The program STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to implement a model-based clustering method to individual genotypes in order to determine the optimal number of populations (K) and examine admixture across the study area (Rosenberg et al. 2002). To determine the optimal K, the number of subgroups (K) was estimated by initiating 20 independent runs of K=1-4 with 10,000 burn-in steps and 10,000 Markov Chain Monte Carlo (MCMC) repetitions as recommended by Evanno et al. (2005). Determining the optimal K minimizes Hardy-Weinberg and linkage disequilibria (Pritchard et al. 2000). These steps were established without *a priori* knowledge of population delineation information, while program parameters were set to their default values as advised by Pritchard et al. (2010). Specifically, I chose the admixture model

and the option of correlated allele frequencies between populations, as this configuration is considered best by Falush et al. (2003) in cases of subtle population structure. Posterior probability was then calculated for each value of K using the estimated log-likelihood of K to select the optimal K (Evanno et al. 2005). I chose the number of clusters suggested by inflection in the rate of change in log probability of successive K values (ΔK). Once K was chosen, individuals were assigned to each of the K groups based on sample locality and its relation to the apparent shift in population affinity determined by STRUCTURE.

The genetic structure of the tentative populations was examined using a number of exploratory and inferential genetic analyses to quantify differences between putative populations and to determine the strength of genetic structure. A factorial Detrended Correspondence Analysis (Lebart et al. 1984) was performed on the multilocus microsatellite genotypes of all individuals using the '2D' 132 module of the software program GENETIX 4.04 (Belkhir 2001) to account for any correlation between genetic distance and geographic distance. A measure of differentiation in allele frequencies, G_{ST} (Hedrick 2005), was derived for all individuals as well as in ecoregion comparisons using GENETIX 4.04 (Belkhir 2001).

RESULTS

One-hundred of 106 samples collected were successfully amplified through PCR and resultant microsatellite tracings were used in analyses. Despite exhaustive extraction and amplification efforts, 6 of 106 samples were not successfully amplified through PCR and could not be included in the analyses. The 6 non-amplified samples were "roadkill"

individuals, leading me to believe that low quality DNA due to decomposition was the limiting factor inhibiting amplification.

Patterns of Genetic Diversity

Multilocus genotypes from 100 samples were included in the analyses. Individuals were assigned to ecoregions based on GPS locations: 56 porcupines in the High Plains, 31 porcupines in the Rolling Plains, and 13 porcupines in the Edwards Plateau (Figure I.3). Samples with fewer than 5 of the loci amplified were not included in the analyses. Four of the 100 porcupines had incomplete genotypes, with 5, 10, 11, and 12 missing loci. The resulting sample set was analyzed as one global population as well as 3 putative populations.

Results examined as one global population (Table I:2): Each of the 17 microsatellite loci was polymorphic with 13 ± 5.82 alleles per locus. Expected heterozygosity ($H_E = 0.8327$) exceeded observed heterozygosity ($H_O = 0.7748$) across all 17 loci, and the phylogenetic information content (PIC) of each locus ranged from 0.567-0.9328. Observed heterozygosity marginally exceeded expected heterozygosity in 6 of 17 loci (Erdo18, Erdo21, Erdo22, Erdo28, Erdo29, & Erdo31) across the population by locus, with differences ranging from 0.003-0.075. Three loci pair comparisons (Erdo7 & Erdo12, Erdo19 & Erdo28, and Erdo24 & Erdo28) were found to be approaching statistical significance ($P = 0.00000$) for linkage disequilibrium following Bonferroni correction ($P < 0.00037$). Six of 17 loci (Erdo7, Erdo11, Erdo19, Erdo30, Erdo40, Erdo42) were found to be out of Hardy Weinberg Equilibrium (HWE) following Bonferroni correction ($P < 0.00294$) (Table I.3). Using GENEPOP, the overall F_{IS} =

0.0682, with higher positive values supporting deviations from HWE in some loci (Erdo11, Erdo40, Erdo42).

Results examined as 3 putative populations (Table I:2): Each of the 17 microsatellite loci were polymorphic across ecoregions with 11.71 $\pm$ 5.35 alleles per locus within the High Plains (HP), 10.24 ± 3.95 alleles per locus within the Rolling Plains (RP), and 7.88 ± 2.64 alleles per locus within the Edwards Plateau (EP). Expected heterozygosity exceeded observed heterozygosity across all 17 loci: H_E = 0.8314 and H_O = 0.7623 within the HP, H_E = 0.8327 and H_O = 0.8018 within the RP, and H_E = 0.8228 and H_O = 0.7602 within the EP. Observed heterozygosity marginally exceeded expected heterozygosity in 6 of 17 loci (Erdo18, Erdo21, Erdo22, Erdo28, Erdo29, Erdo31) across all populations by locus, with differences ranging from 0.0192-0.0621. The PIC of each locus ranged from 0.5722-0.936 in the HP, 0.5828-0.9026 in the RP, and 0.4262-0.8908 within the EP. Only one loci pair comparison (Erdo9 & Erdo31) within the HP was found to be approaching statistical significance for linkage disequilibrium following Bonferroni correction (α = 0.00012). Four loci (Erdo11, Erdo19, Erdo40, Erdo42) in the HP, 2 loci (Erdo40, Erdo42) in the RP, and 1 loci (Erdo40) in the EP, were found to be out of Hardy Weinberg Equilibrium (HWE) following Bonferroni correction ($P <$ 0.00294) (Table I.3). Using GENEPOP, an overall F_{ST} = 0.0022 across all 3 ecoregions suggested minimal structure and little divergence within the sample set. The overall F_{IS} = 0.0682, with higher positive values supporting deviations from HWE in some loci (Erdo11, Erdo40, Erdo42). An overall G_{ST} value of 0.0019 was obtained for porcupines across all ecoregions, suggesting that panmixia may be widespread throughout western Texas because of low variation of allele frequencies.

Population Structure

The Bayesian analysis identified subtle population structure. A mode was observed at K =2 (Figure I.4), but was similar to the likelihood at K =1 and unlike analyses employing K= 4 or 5. The mode at K = 3 was weak (Figure I.5). The STRUCTURE analysis (Pritchard et al. 2000) found no distinct population subdivision correlated to geographic location, with porcupines being equally likely to assign to cluster 1 in the High Plains as porcupines assigning to cluster 1 in the Rolling Plains. When K =2 for the total sample, 51.4% of porcupines identified most strongly with cluster 1, while 48.6% identified most strongly with cluster 2 (Figure I.4). When K = 3, 32.5% of porcupines identified most strongly with cluster 1, 34.2% identified with cluster 2, and 33.2% of porcupines in cluster 3 (Figure I.5). Genetic discontinuity did not correspond to sample location or to ecoregion with any putative population simulation.

The factorial Detrended Correspondence Analysis for all samples (Figure I.6) found little structure across one global population, as well as across 3 putative populations within ecoregions, with 6 outliers not falling within the single cluster determined by GENETIX (Belkhir 2001). Outliers were isolated points outside of the main cluster and were not associated with a second cluster. Four outliers were located in the High Plains, 2 in the Rolling Plains, and 0 in the Edwards Plateau ecoregion. To more clearly identify fine-scale differentiation in the main cluster, these outliers were removed from the dataset and the analysis was run again. The correspondence analysis for the remaining 94 samples again detected one primary cluster, but some weak ecoregion affinity was present because of animals plotting nearer to animals from the same ecoregion (Figure I.7). Porcupines from the High Plains, however, exhibited the

greatest genetic variability and were evenly distributed throughout the primary cluster. To examine the clustering relationship among the samples with less genetic variability, I performed this analysis a third, fourth, and fifth time, each run sans outliers as well as all samples from the High Plains, Rolling Plains, and Edwards Plateau ecoregion (respectively). Subsequent analyses revealed a weak trend for ecoregion affinity, but little structure was still apparent in the clustering.

DISCUSSION

The variation in multilocus microsatellite genotypes for 100 porcupines sampled throughout 3 ecoregions in western Texas document minimal genetic structure throughout the study area. Overall expected heterozygosity exceeded observed heterozygosity. This finding was supported by the homozygote excess identified with a moderate overall inbreeding coefficient (F_{IS} = 0.0682), where a heterozygote deficiency suggested possible population subdivision. The genetic structure throughout western Texas, as evidenced by the likelihood that K =1 and K = 2 in the STRUCTURE analysis, was weak (Figure I.4). For example, some porcupines in the Edwards Plateau ecoregion exhibited the same likelihood of falling into cluster 2 as individuals from the High Plains ecoregion, which is physically separated from the High Plains by the Rolling Plains ecoregion. When I mapped porcupines geographically by population from STRUCTURE, however, there was no geographic pattern when K = 2 or K = 3 (Figure I.5). Further, the Detrended Correspondence Analysis illustrated a primary genetic cluster with minimal grouping by ecoregion (Figure I.6).

The number of alleles per locus (k) suggests that polymorphism, the allele's ability to exist in several different forms, is present within the population. Overall

expected heterozygosity exceeded observed heterozygosity, implying a moderate level of genetic diversity across all loci. The 6 individually analyzed loci with an observed heterozygosity that exceeded expected heterozygosity, revealed a small margin of difference that had no effect on overall measures of genetic variability. These values all support the notion that this highly mobile species is able to move throughout its habitat to successfully promote gene flow.

When analyzed as 3 separate populations, an overall G_{ST} value of 0.0019 was obtained for porcupines across all ecoregions, suggesting that panmixia may be widespread throughout western Texas because of low variation of allele frequencies. Within ecoregions, low variation of allele frequencies for porcupines in western Texas also suggested little to no substructuring. Heterozygote deficiencies compared to HWE may indicate that porcupines are exhibiting assortive nonrandom mating, where individuals with dissimilar genotypes and/or phenotypes mate with one another more frequently than would be expected under random mating. Nonrandom mating can change genotype frequency but cannot change allele frequency. It seems unlikely that this notion supports the genetic diversity and moderate overall inbreeding coefficient observed across the study area. Alternatively, heterozygote deficiencies compared to HWE may indicate heterozygote alleles are being expressed as a homozygote allele, as is supported by some loci (Erdo11, Erdo19, Erdo40, Erdo41). This scenario is more likely as both the Bayesian and Detrended Correspondence Analysis of this data set imply porcupines belong to a panmictic population (large randomly mating population with no substructure).

Alleles at most loci in large random mating populations are in linkage equilibrium. Loci showing deviations from this equilibrium in large random mating populations may be subject to natural selection (Frankham et al. 2007). The three loci comparisons found to be approaching statistical significance for linkage disequilibrium in one global population did not share a common locus, and are not likely under selection. With 100 animals examined across 17 polymorphic loci, some linkage is expected, but does not affect the overall population structure.

Identifying the variables involved in structuring populations are challenging in abundant, widely distributed species, particularly if they are wide-ranging, highly mobile, and/or generalists. The evolution of a species across large distances likely results in several factors, sometimes making discernment of the causes of genetic structure complex. For highly-mobile species, gene flow is not expected to be limited by environmental features because of the ecology and dispersal abilities of these organisms; populations are often genetically panmictic, where dispersal is not geographically limited and unrestricted gene flow limits the development of population genetic structure (Wright 1943). Populations of all sexual species exhibit some individual variation, but it can be difficult to determine what myriad of factors may be influencing the structure and genetic variation of a population. The shared population metrics of this sample set imply a sole source population upon arrival into the Davis mountains habitat. Naturalists (Bailey 1905) speculate northern Mexico as the source population, but no genetic evidence exists for this hypothesis. A founder effect, or the change in genetic composition of a population due to origin from a small sample of individuals (Frankham et al 2007), may have resulted in a loss of genetic diversity, loss of alleles and an increase in inbreeding

overtime within the sample set. Hoffer (1967) reported temperature, vegetative types, and estrus directly affected porcupine movement, while precipitation, topographic features and animal age did not appear to limit their movement. It is most likely that the porcupine's relatively recent establishment into south Texas (Bailey 1905) and subsequently north into western Texas (Dalquest and Horner 1958) is the basis of the lack of structure within the sampled population.

A consideration for the results of this study is that the sample size and distribution was inadequate to delineate meaningful insight into the population structure of porcupines in western Texas. Specifically, more samples from the Edwards Plateau would be beneficial. The low sample yield from this ecoregion (n = 13) makes identifying structure weak. Samples were comprised primarily of "roadkill" samples collected opportunistically across the study area. A more strategic and study site specific method of sample collection may have produced different population structuring observations and is recommended.

Overall, my research reveals that porcupines throughout western Texas are indeed vagile. The lack of population structure found in western Texas is likely the result of the relatively short life history of this highly mobile species within the state of Texas. The moderate genetic diversity reflects porcupine's wide use of habitat throughout the western portion of the state. More research is needed in other porcupine populations across other habitats to better support the findings of this research. This knowledge is beneficial in the management of this species, considered a pest by some, and to the overall understanding of the porcupine.

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Locus	Primer Sequence 5'→3'	Size (bp)
Erdo7	*F: TTGGAATGGTCATTTGTGTGG**	322-346
	R: AGAGCTTTCATGTTGAGCTGC	
Erdo9	*F: CACTGGGAGGTTGTTAGCC**	220-270
	R: AGGACACATCAACATAAGCACC	
Erdo11	*F: TACATAGCAAGACCCGACCC**	124-152
	R: CAAGTGGTTTAGCACTGGGC	
Erdo12	*F: AAACAGAACCCAGAATTGTCATATACC**	196-228
	R: CACACTGCTAGGATGTTGTCTCC	
Erdo14	*F: AAATAAAGTTTACAAGCCAAAGCC**	296-374
	R: CATGACTAAGTACATCCCAATTTCC	
Erdo18	*F: CATTGCAATCGTCAGAGAGG**	196-228
	R: CCTGTGTCTCCATCTTGGC	
Erdo19	*F: ATTACAGAGAAATGGAAATGTAATCC**	166-194
	R: CTATGTGTTTAATCTCCAGTACCACC	
Erdo21	*F: CCCATACCCACACACACG**	220-260
	R: CTGAGACCCATTAGCTTGGC	
Erdo22	*F: GGGAAGCACAATGTAGGATGC**	201-276
	R: CTGTTCTGGTCTCAGCGTGG	
Erdo24	*F: CCACTGCCTGGACTTGAAGC**	306-334
	R: GATGTCCTGAGCCTGGTGC	
Erdo28	*F: GAGAGTGGACAAATTTATGATTACATAGG**	176-236
	R: GGCTGGGAGTGTAGCTGAGG	
Erdo29	*F: TGTTCTGGGAAATTGATAAGTAGCC**	142-162
	R: GGGTCTTGCCTCAGTAAAGGG	
Erdo30	*F: TTCCACTCCAGGACATTCCC**	367-411
	R: GTCACCATTAGAATCTCTGCTGC	
Erdo31	*F: GGGTAGCATGAGGGTATAGAGC**	188-213
	R: GTGTAGCCACACAGGCAGG	
Erdo32	*F: GCTGCAATCAAAGACAAGCC**	204-236
	R: TAAGGGCCCATTAGCTGGC	
Erdo40	*F: CCAAAGCCTTCGGACAAAGC**	198-210
	R: TGTAATAAGGGACGGGATTAAATTATGG	
Erdo42	*F: TGTAGATACAGATCAGCCAATAGGG**	220-244
	R: TTCACCAACTGTGAACATTTCC	

Table I.1: Details for 17 polymorphic microsatellite loci developed by Barthelmess (2013) for use in population genetics of the North American porcupine (*Erethizon dorsatum*). * *Indicates* D4-PA tag (5'-GAGTTTCCCAGTCAC-3') label.

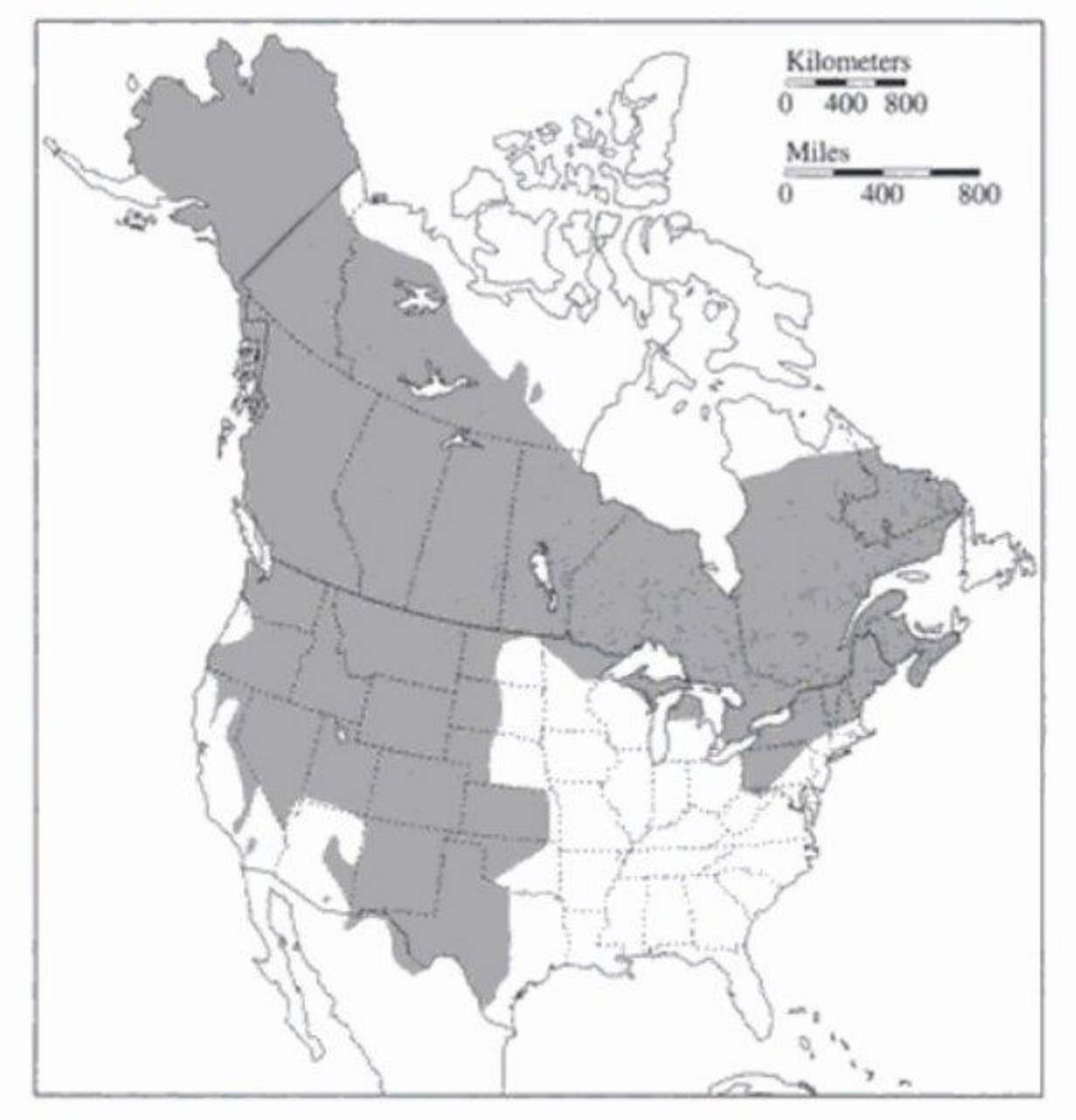

Figure I.1: Distribution map of the North American porcupine. Map Credit: Roze and Ilse (2003).

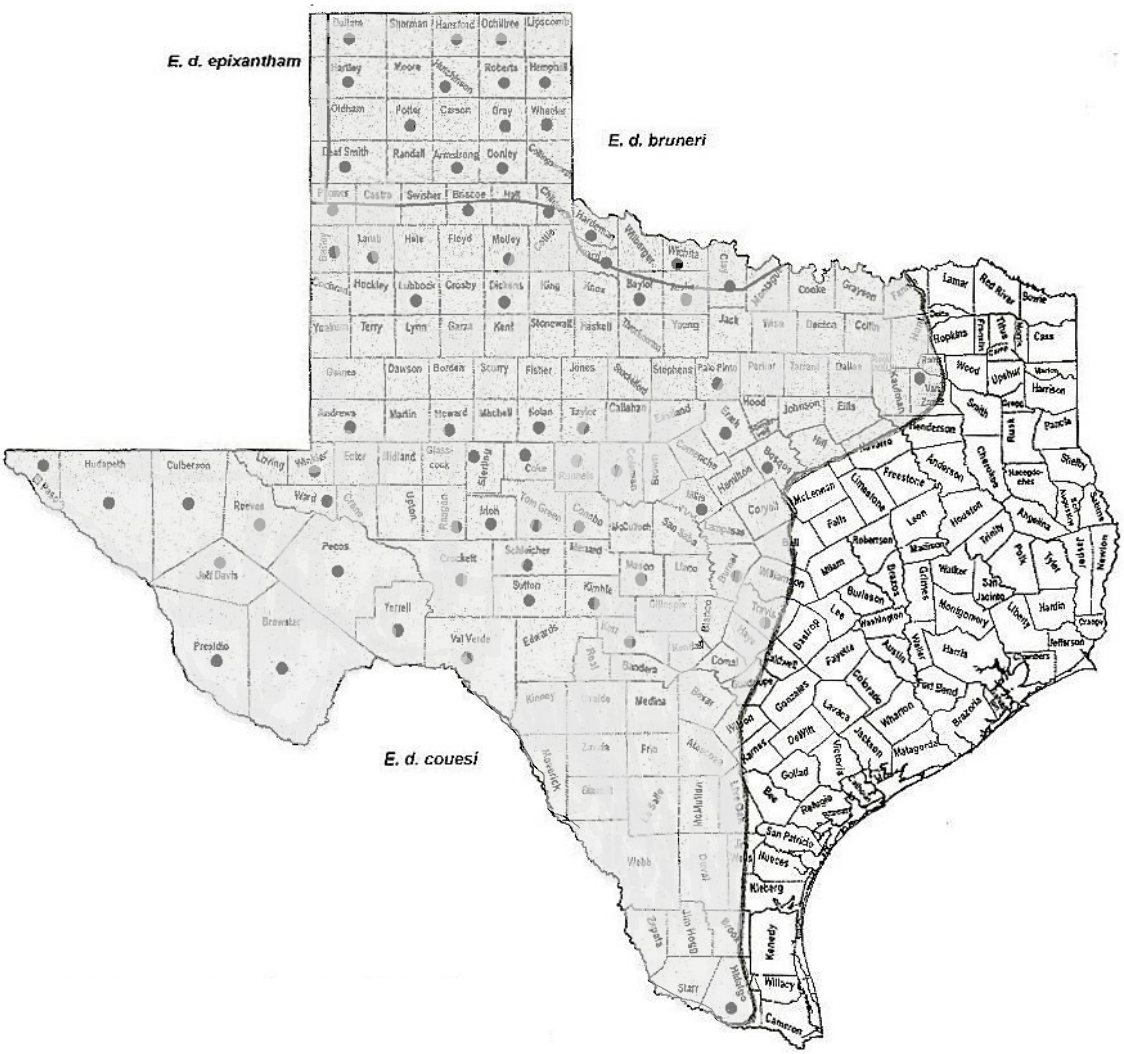

Figure I.2: Distribution of the North American porcupine in Texas based on known county records. Shaded portion of the map represents current distribution. Black lines represent distribution of 3 recognized porcupine subspecies found within Texas: E. d. *epixantham*, E. d. *bruneri*, and E. d. *couesi*. Map Credit: Schmidly and Bradley (2016).

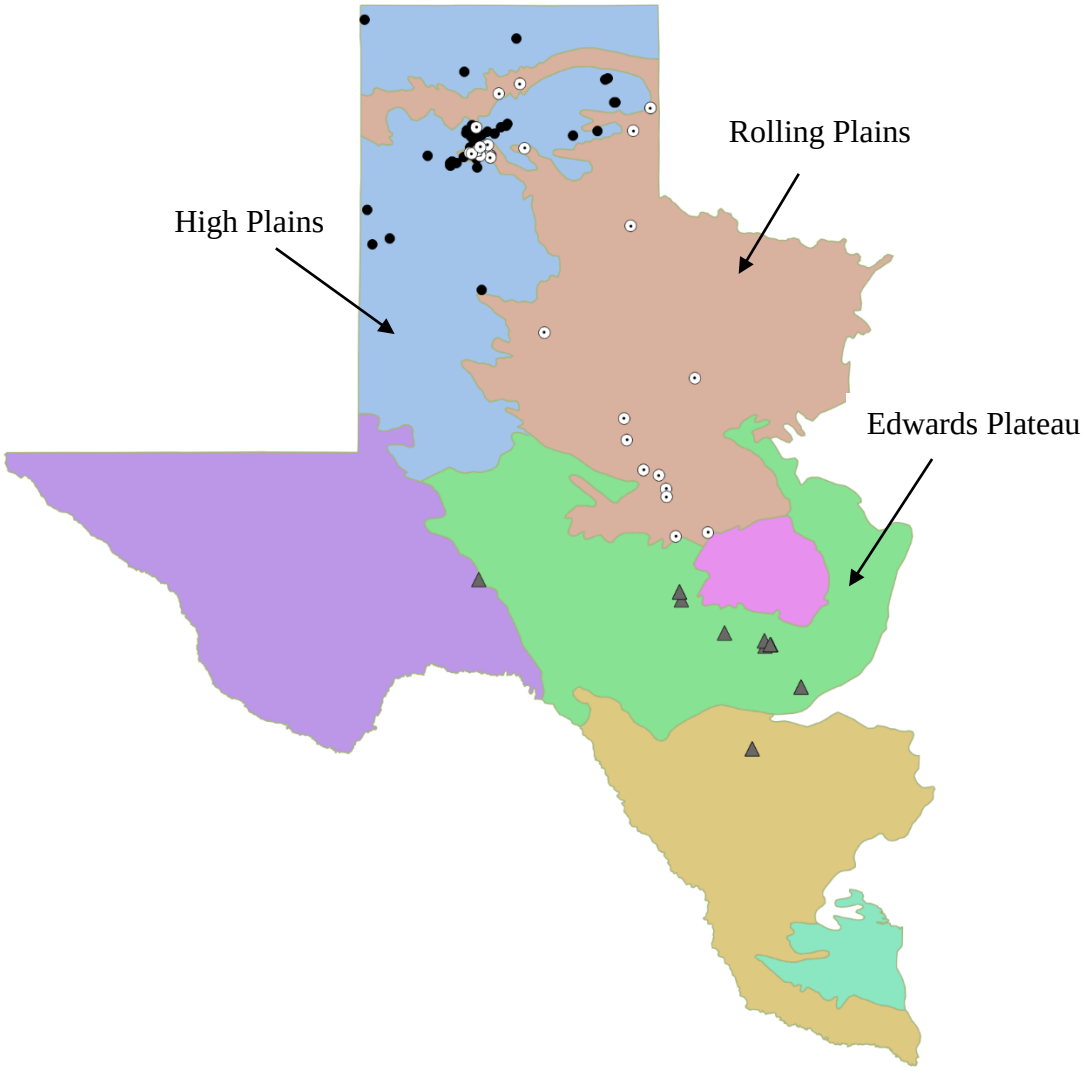

Figure I.3: GPS locations of 106 porcupine samples from 3 putative populations across 3 ecoregions in western Texas. Black circles = High Plains porcupines, white circles = Rolling Plains porcupines, and gray triangles = Edwards Plateau porcupines.

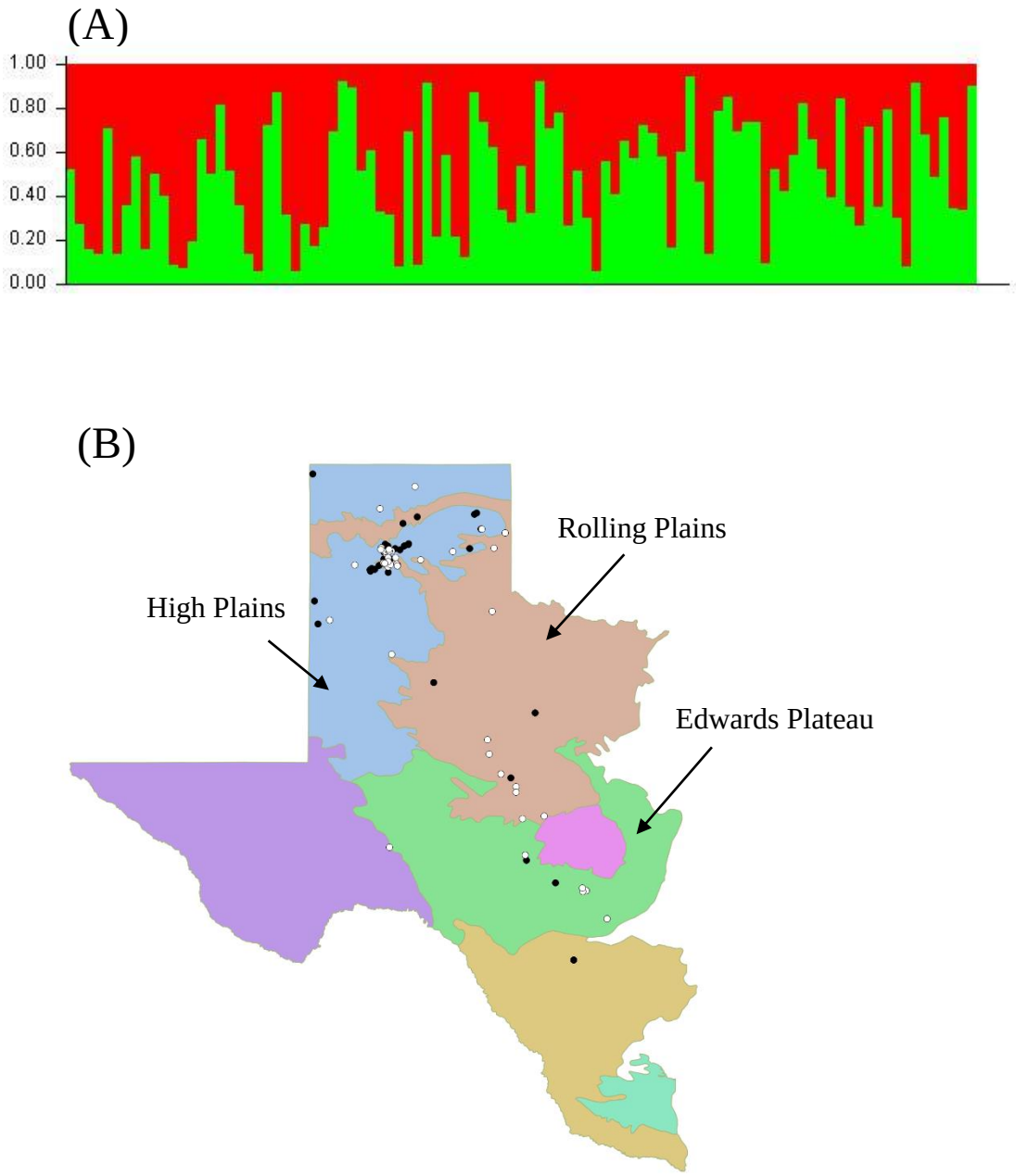

Figure I.4: Porcupine populations when K =2 from the STRUCTURE analysis (A) and when organized by inferred populations across the study area (B). Black circles = population 1 and white circles = population 2. See text for full description.

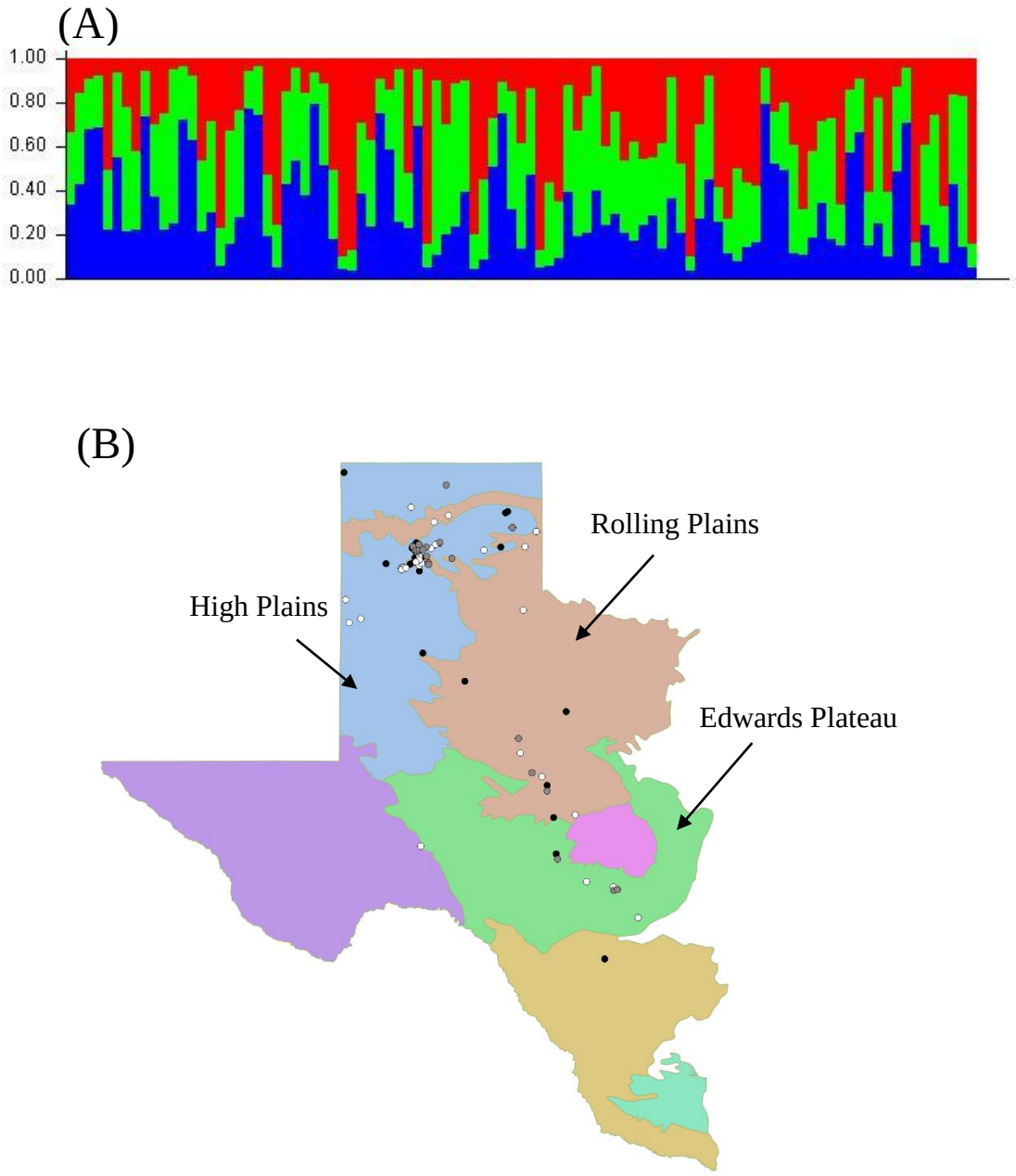

Figure I.5: Porcupine populations when K =3 from the STRUCTURE analysis (A) and when organized by inferred populations across the study area (B). Black circles = population 1, white circles = population 2, and gray circles = population 3. See text for full description.

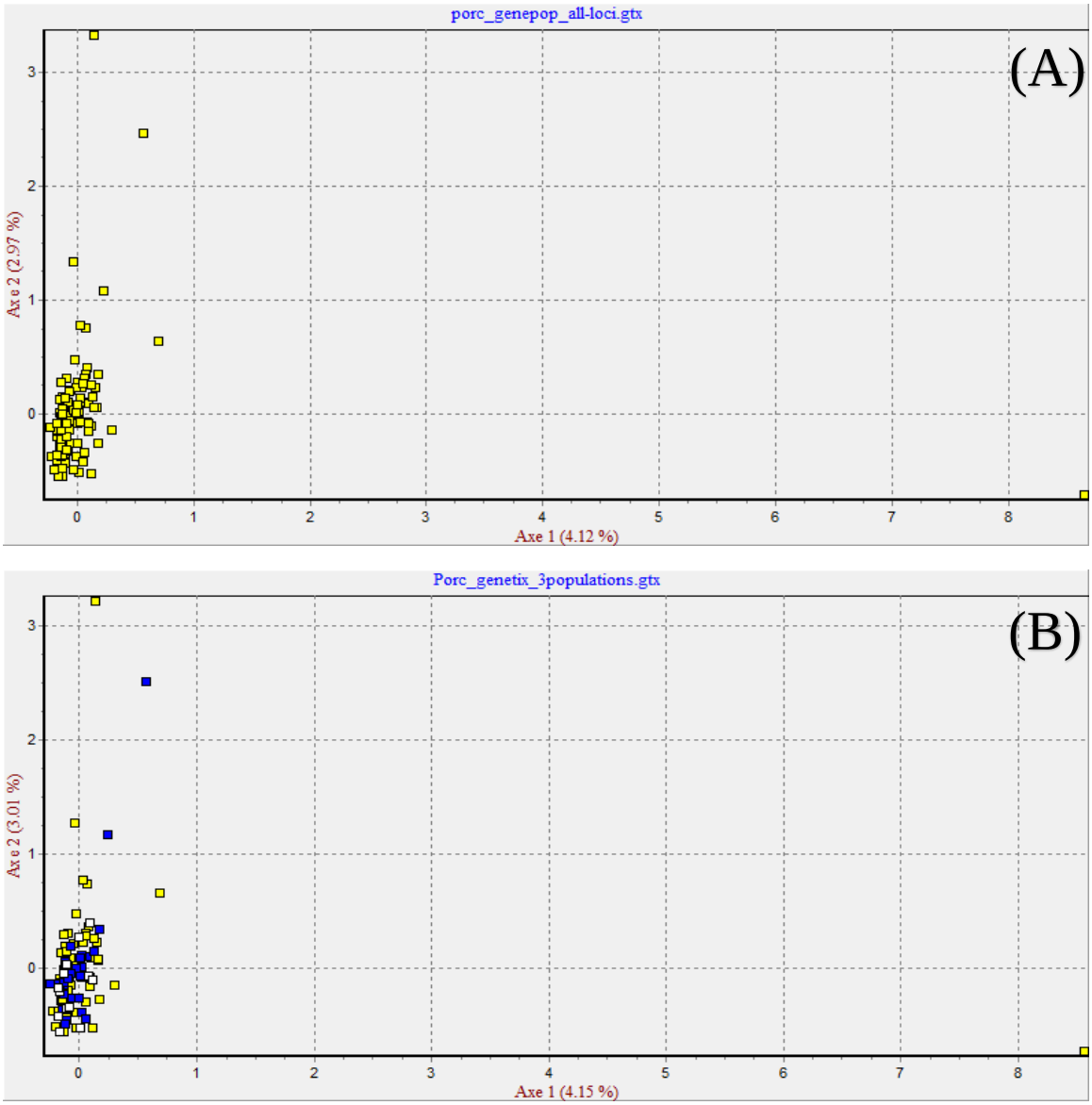

Figure I.6: Results of the Detrended Correspondence Analysis with all porcupines. Porcupines fell into 1 cluster when analyzed as 1 global population (A) and as 3 putative populations (B) and are individually represented by colored squares. Yellow = one global population (A) & High Plains porcupines (B), Blue = Rolling Plains porcupines, White = Edwards Plateau porcupines.

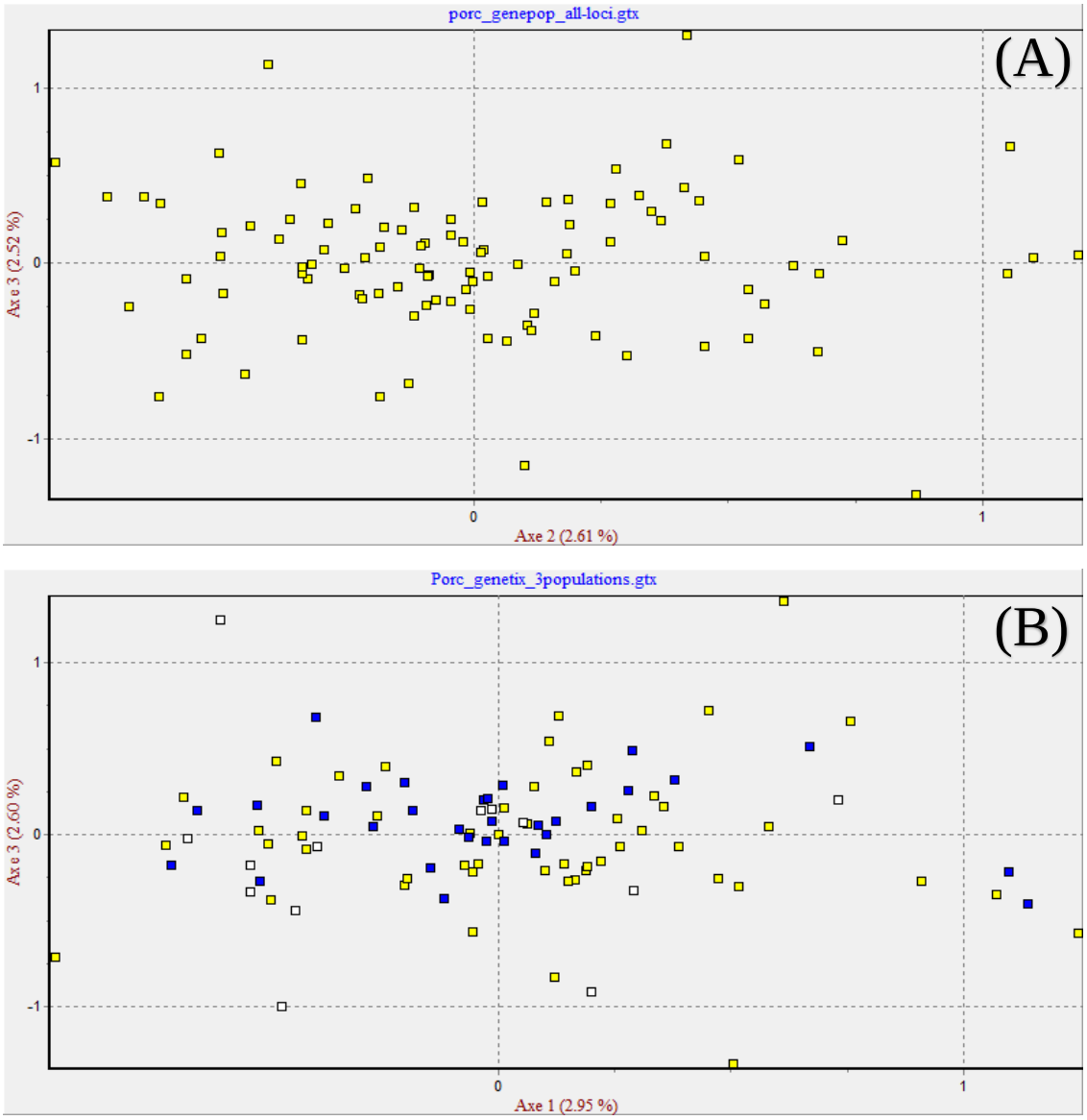

Figure I.7: Results of the Detrended Correspondence Analysis sans outlier porcupines. Porcupines fell into 1 cluster when analyzed as 1 global population (A) and as 3 putative populations (B) and are individually represented by colored squares. Yellow = one global population (A) & High Plains porcupines (B), Blue = Rolling Plains porcupines, White = Edwards Plateau porcupines.

Measures of Genetic Variation

Population	N	A_N	H_E	H_O	PIC			
Global	100	13.12	0.8327	0.7748	0.567-0.933			
						F_{IS}	**F_{ST}**	**G_{ST}**
High Plains	56	11.71	0.8314	0.7623	0.572-0.936			
Rolling Plains	31	10.24	0.8327	0.8018	0.583-0.903			
Edwards Plateau	13	7.88	0.8228	0.7602	0.426-0.891			
						0.0682	0.0022	0.0019

Table I.2: Details of population genetic metrics within the sample set analyzed as one global population and as three populations across the study area. Number of individuals genotyped is *N*; average number of alleles is A_N; H_E and H_O are expected and observed heterozygosity, respectively; *PIC* is the range of polymorphic information content; F_{IS} and F_{ST} are inbreeding coefficient and population subdivision, respectively; G_{ST} is the differentiation in allele frequencies.

Hardy Weinberg equilibrium

P < 0.00294	**Global**	**High Plains**	**Rolling Plains**	**Edwards Plateau**
Locus7	0.0028			
Locus11	0.0000	0.0000		
Locus19	0.0000	0.0011		
Locus30	0.0000			
Locus40	0.0000	0.0000	0.0000	0.0000
Locus30	0.0000			
Locus42	0.0000	0.0000	0.0000	

Table I.3: Details for deviations from Hardy-Weinberg equilibrium within the sample set analyzed as one global population and as three populations across the study area. P-values for loci shown indicate significant deviations from Hardy–Weinberg expectations after Bonferroni corrections ($P < 0.00294$).

CHAPTER II

MOLECULAR SEX IDENTIFICATION OF PORCUPINES IN WESTERN TEXAS

Introduction

An animal's mating system often reflects the sex ratio of a population and whether dispersal habits are male- or female-biased. The North American porcupine (*Erethizon dorsatum*) exhibits a polygynous mating system in which females only obtain one male and dominant males seek to obtain several females per breeding season. This type of polygyny would assume a male-biased natal dispersal pattern. Males invest no parental care in offspring and gain most from dispersal, while females invest heavily and should gain most from being sedentary (Greenwood 1980, Dobson 1982). Dispersal is male-biased throughout the majority of mammals, with only a few species having been observed as female-biased (Greenwood 1980). A variety of ecological and behavioral hypotheses have been proposed to account for differences in sex-biased dispersal, including: population density, avoidance of inbreeding, access to mates, access to resources, and reproductive enhancement (Bengtsson 1978, Greenwood 1980, Sweitzer and Berger 1998).

Roze (1989), Sweitzer and Berger (1998) and Ilse and Hellgren (2001) provide compelling evidence suggesting an atypical female-biased natal dispersal pattern in the

North American porcupine. These results contradict the contention that mating systems often predict dispersal patterns and that in taxa exhibiting polygamous mate-defense, the limited sex is likely to disperse. Roze (1989) suggested the female-biased sex ratio and increased resource demand on females during gestation and lactation provide probable cause for this anomaly, whereas Sweitzer and Berger (1998) indicate that avoidance of inbreeding provides the strongest explanation. Dispersal in porcupines is not well researched, but evidence thus far supports the idea that it is female-biased.

Porcupines exhibit mate-defense polygyny whereby males, responding to olfactory and auditory signals of pre-estrous females, engage in active defense and competition (Sweitzer and Berger 1998). Reproductive success of males depends on the number of females they are able to monopolize during the mating season. Adult females defend relatively small exclusive territories, while adult males occupy large undefended home ranges (Roze 1989). Large breeding period home ranges of dominant male porcupines are positively correlated to mating success, often leading males to travel over large areas in search of females (Sweitzer 2003).

Demographic studies of North American porcupine populations typically reveal a female-biased sex ratio (Hale and Fuller 1996, Krefting et al. 1962, Roze 1989, Sweitzer and Berger 1998), with few reporting an excess of males occurring inside the study area (Hoffer 1967, Randall 1971). Within the Edwards Plateau and High Plains ecoregions of Texas, Ilse and Hellgren (2001) observed a female-biased adult sex ratio of 17 females to 5 males and D. Montgomery (West Texas A&M University, unpublished data) documented a female-biased adult sex ratio of 14 females and 1 male, respectively.

The North American porcupine does not readily exhibit external sexual traits aside from observed pregnancy and breeding behavior, making sex determination by visual inspection difficult (Shadle 1950). Unequivocal sexing can be achieved by abdominal palpitation for the presence of the penis (Mirand and Shadle 1953), but often requires chemical immobilization due to the inherent difficulty in handling and restraint of this species (Schroeder and Robb 2005).

Sexing of individuals in mammalian populations is essential for understanding population dynamics, management decisions, population structure and habitat use, sex ratio of social groups, and mating systems (Shaw et al 2003). Molecular sex determination is essential in situations when the animal is not present, but when tissues are available or sex-specific characters are either absent of difficult to observe (Griffiths and Tiwari 1993). Two key molecular techniques have been developed for DNA sex determination in mammals. The first amplifies the Y-specific (male) fragments of the sex determination region (SRY) gene (Griffiths and Tiwari 1993). Nonamplification of the target fragment thus equates with female identity. However, the lack of an SRY amplification product may not indicate the lack of a Y-chromosome but instead a lack of PCR (polymerase chain reaction) effectiveness (Griffiths and Tiwari 1993). This method has been used to determine sex in a variety of mammalian species from marsupials (Bianchi and Bianchi 1993) to cetaceans (Palsbøll et al. 1992).

Alternatively, the analysis of sex-linked zinc finger (ZF) protein genes, which are present in both female and male chromosomes, can be used for sex determination (Ortega et al. 2004, Xu et al. 2010). Discrimination of sex is based on either the presence of a heterozygous zinc finger sequence for males (Zfx and Zfy) or the homozygous zinc

finger sequence for females (Zfx) (Ortega et al 2004). The amplification of 2 differently-sized products, 1 from the X chromosome and 1 from the Y chromosome, is a substantial benefit of this method over previously used methods (Shaw et al. 2003). By developing a single, often species-specific, primer pair this method has been used to determine sex across a multitude of mammalian species including the harbor seal (*Phoca vitulina*), moose (*Alces alces*), black bear (*Ursus americanus*), coyote (*Canis latrans*) (Shaw et al. 2003), giant panda (*Ailuropoda melanoleuca*) (Xu et al. 2010), beaver (*Castor canedensis*) (Kühn et al. 2002), and white-tailed deer (*Odocoileus virginianus*) (Lindsay and Belant 2008).

The ZF method has recently been developed for use in the prehensile-tailed porcupine (*Coendou prehensilis*) by directly sequencing a short portion (195 base pairs) of the ZF protein gene of known male individuals to identify positions that are polymorphic between the X and Y chromosomes at this locus (Woc-Colburn et al. 2013). Woc-Colburn et al. (2013) reported this 195-bp fragment of ZF sequence as being fairly conserved within New World porcupine species by revealing 16 base pair substitutions between Zfx and Zfy chromosomes in both the prehensile-tailed and North American species (Table II.1). Woc-Colburn et al. (2013) also successfully applied this method to 3 North American porcupines and recommends further testing with larger sample sizes to confirm its applicability.

The objectives of this study were to confirm the applicability of the zinc finger protein sequencing method to determine sex in a population of North American porcupines and to observe the male/female presence of porcupines across 3 ecoregions of western Texas. I hypothesized that generated sequencing data would correctly identify

sex of individuals within my sample set and, based on prior reports, that more females than males would be observed across the study area.

METHODS

Study Area and Sample Collection

One hundred six porcupine tissue samples were opportunistically collected from September 2013 to December 2016 throughout portions of the High Plains, Rolling Plains, and Edwards Plateau ecoregions of western Texas (Fig. II.1).

All capture methods and handling procedures met the requirements of the Guidelines for the Capture, Handling, and Care of Mammals as approved by The American Society of Mammalogists and West Texas A&M University Standard Operating Procedures SOP No. 15.99.05.W1.02AR Institutional Animal Care and Use (IACUC# 3-10-13). Porcupines were captured using 2 methods: Tomahawk wire live traps (106.7 cm X 30.5 cm X 30.5; Tomahawk Live Trap, Tomahawk, WI) were baited with apples and salt and placed at the ends of culverts and the bases of occupied trees (Griesemer et al. 1999), and porcupines found on the ground were captured by hand using Kevlar-lined leather gloves (Shadle 1950). GPS coordinates were recorded for each individual (O'Neil et al. 2005), and anatomical observations were recorded via photographic evidence and/or the 'Apple' application Theodolite (Hunter Research and Technology L.L.C.; accessible at hunter.pairsite.com/theodolite).

For live-trapped individuals, tissue samples collected for DNA analysis included quills with attached root follicles and fecal material. Animals were released at the site of capture. For individuals of vehicular casualties, postmortem tissue samples included

hairs and quills with attached root follicles and muscle tissue. Hair, quill, and muscle tissues were stored either dry and frozen at -20 °C or submerged in 99% ethanol at room temperature. Fecal samples were stored frozen at -20 °C.

DNA Extraction and Sequencing

Genomic DNA was extracted from fecal matter using the FastDNA® Sample Spin Kit Feces (MP Biomediacals, LLC, Solon, OH.) following the manufacturer's instructions. Genomic DNA extractions were performed on hair, quill, and muscle tissues using a modified DNeasy® Blood and Tissue Kit (Spin-Column Protocol) (QIAGEN Inc., Valencia, CA, USA) (Woc-Colburn et al. 2013): centrifuge rpm and speed was modified during step 6 from the protocol's recommended 14,000 rpm for 3 minutes to 13,4000 rpm for 4 minutes due to limited rpm capabilities of available centrifuges, and the amount of elution buffer (Buffer AE) from step 7 was modified from the protocol's recommended 200 µL per sample, based on the detected concentration of DNA after an initial elution of 50 µL. DNA with a low concentration (<10 ng/µL) was kept at an elution volume of 50 µL and DNA with a higher concentration (>10 ng/µL) was eluted to volumes of 50-200 µL as needed. For all samples, DNA concentration was detected using a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a Qubit™ dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Genomic DNA was maintained at 2.7 °C for immediate use or stored at -20 °C for future use.

One hundred and six porcupines were amplified with a 195 base-pair fragment of the sex-linked zinc-finger (ZF) protein gene, Zf*x* on the X chromosome (forward ZFKF 203L 5'CAAAAGGTGGCGATTCAATAA-3') and Zf*y* on the Y chromosome (reverse

ZFKF 195H 5'-ATGGAGAGCCACAAGCTRAC-3') developed by Ortega et al. (2004). All initial polymerase chain reaction (PCR) amplifications were performed in a 25.0µL volume with final reagent concentration and volumes as follows: 12.5µL of GeneMate Red Taq 2x Master Mix (BioExpress, Kaysville, UT, USA), 2.0 µL of 100.0 mg/mL bovine serum albumin (BSA), 1.5µL of 10µM Zf*x* primer (Sigma-Aldrich Inc., St. Louis, MO, USA), 1.5µL of 10µM Zf*y* primer (Sigma-Aldrich Inc., St. Louis, MO, USA), 20ng template DNA, and ddH_2O. PCR conditions were an initial denaturation step of 5 min at 95°C, 35 cycles of 60 sec at 94°C, 60 sec at 58°C, 90 sec at 72°C, and a final extension step of 72°C for 5 min. All amplifications were performed on an Eppendorf Mastercycler® Gradient 5333 (Eppendorf, Hamburg, Germany). PCR amplicons were run for 45 min at 100 V in a 1% agarose gel stained with 10mg/mL ethidium bromide and visualized under UV light to determine successful amplification. Four µL of GeneMate100 bp Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) was included on each gel for each reaction as a size standard. Negative controls (no sample material added to the extraction) accompanied each set of PCR amplicons to check for possible contamination. DNA from porcupines of known gender accompanied each set of PCR amplicons as a positive control.

Because of their high-resolution output and extremely cost-effective services, the resulting 106 PCR amplicons were then transferred to the University of Arizona Genetics Core sequencing facility (UAGC 2017). An ExcelaPure PCR purification system (Edge BioSystems, Gaithersburg, MD, USA) was used to purify all amplicons. Amplicons were then quantified and normalized using an Agilent High Sensitivity DNA Kit on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Sequencing was then

completed using the BigDye Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All chemicals, protocols, and equipment were used according to the manufacturer's instructions and without modifications. Chromatograms were aligned and analyzed using the program SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

RESULTS

Of the 106 samples submitted for sequencing, 92 were successfully sequenced and base pair profiles were used to determine sex; 51 identified as male and the remaining 41 identified as female (Table II.2). All known sex samples were correctly assigned to the accurate sex based on the generated sequence data. Chromatograms of sequencing results available upon request.

Variations produced within my sample set agree with that of Woc-Colburn et al (2013) by means of a heterozygous variation in position 31 of A/C and no, or homozygous, variation in position 38 of T/T. In contrast, variations produced within my sample set also revealed that variation site 50 was found to be positioned at 49, variation site 88 was an A/G variation rather than A/T, and variation site 155 was a T/A variation rather than an C/A (Table II.1).

Due to technical limitations in detection capabilities, 16 samples had 1-8 of 16 undetermined base pair variations, resulting in an incomplete sequencing profile. Gender was still determined in all 16 of the incomplete profiles based on a call consensus of the variation pattern present along with anatomical evidence. DNA concentration did not appear to be a contributing factor of an incomplete profile as concentration values varied

from 0.466ng/mL to >600ng/mL. Due to technical limitations in detection capabilities, 6 samples had ambiguous calls at some variations, making it difficult to discern a heterozygous call from a "messy" sequence. Gender was determined in all 6 samples based on a consensus call of the variation patterns present along with anatomical evidence. Due to poor chemical quality, 14 samples were not successfully sequenced either direction. Gender was determined in 6 of the unsequenced samples based on anatomical evidence, leaving 8 samples of unknown gender within the sample set. Budgetary limitations within this study did not allow for re-sequencing of these samples. Acquisition of quality chemicals and repeat sequencing is highly recommended.

Overall, through a combination of sequencing and anatomical evidence, sex was determined for 98 of 106 individuals; 53 male and 45 female. The male/female observance across the 3 ecoregions is as follows: High Plains, 22 male and 29 female; Rolling Plains, 19 male and 10 female; Edwards Plateau,7 male and 6 female (Table II.3). Photographic evidence used to document anatomical evidence of sex available upon request.

DISCUSSION

Based on described sex ratios and mating behaviors, the relative balance in the numbers of male (n=53) and female (n=45) porcupines observed across the 3 ecoregions of this study was very unexpected and contradicted the initial hypothesis that a greater number of females than males would be observed. Because all anatomically confirmed samples were correctly assigned to the known sex based on the generated sequence data, inaccurate sequencing results are highly unlikely to have skewed results in favor of males over females.

Over the 4-year sampling period, 88 of the 98 accurately sexed individuals were collected during the breeding season from late August until November (Dodge 1967). Porcupines consume a wide variety of herbaceous vegetation, woody shrubs, and tree bark throughout the year, but the breeding season and the onset of winter exacerbate movements to obtain said nutritional resources (Dobson 1982). Higher incidences of male mortality via vehicular collisions as a result of increased home range movements by dominant males and subordinate, sexually mature males in search of mating opportunities during breeding season is also a probable cause for observing more males than expected within the study area. This study is lacking in morphological and age data to fully support this notion and warrants a more thorough investigation of sex ratio of porcupines in western Texas. This study was also not conducted to methodically or statistically examine porcupine demography, and samples were primarily collected opportunistically postmortem, rather than strategically, across the 3 ecoregions. Fourteen (13.72%) of the 98 accurately sexed individuals were live capture samples, all other samples (86.28%) were postmortem samples collected from roadsides. The 14 live capture samples consisted of 8 females and 6 males. The remaining 84 postmortem samples consisted of 37 females and 47 males. Sampling efforts suggest that owing to their movements, especially during the breeding season, males are more likely than females to be collected along roadsides within the study area, and females are more likely than males to be collected during live capture efforts. Live capture sampling over a smaller, more specific area or population during times of movement may yield the expected female bias.

The results of this study offer further confirmation that a zinc finger protein sequencing method developed for sex determination in the prehensile-tailed porcupine

can be used to accurately determine sex in populations of North American porcupines. PCR protocols, reagent volumes and concentrations, zinc finger protein primer sequences, and visualization methods can be confirmed for use in *Erethizon* species. This confirmation provides an accurate and non-invasive technique to determine sex *ex situ* from potentially limited and often degraded tissue samples, ensures accuracy when sexing very young individuals that do not yet exhibit breeding behavior or sexual maturity, and allows for better assessment of porcupine demography.

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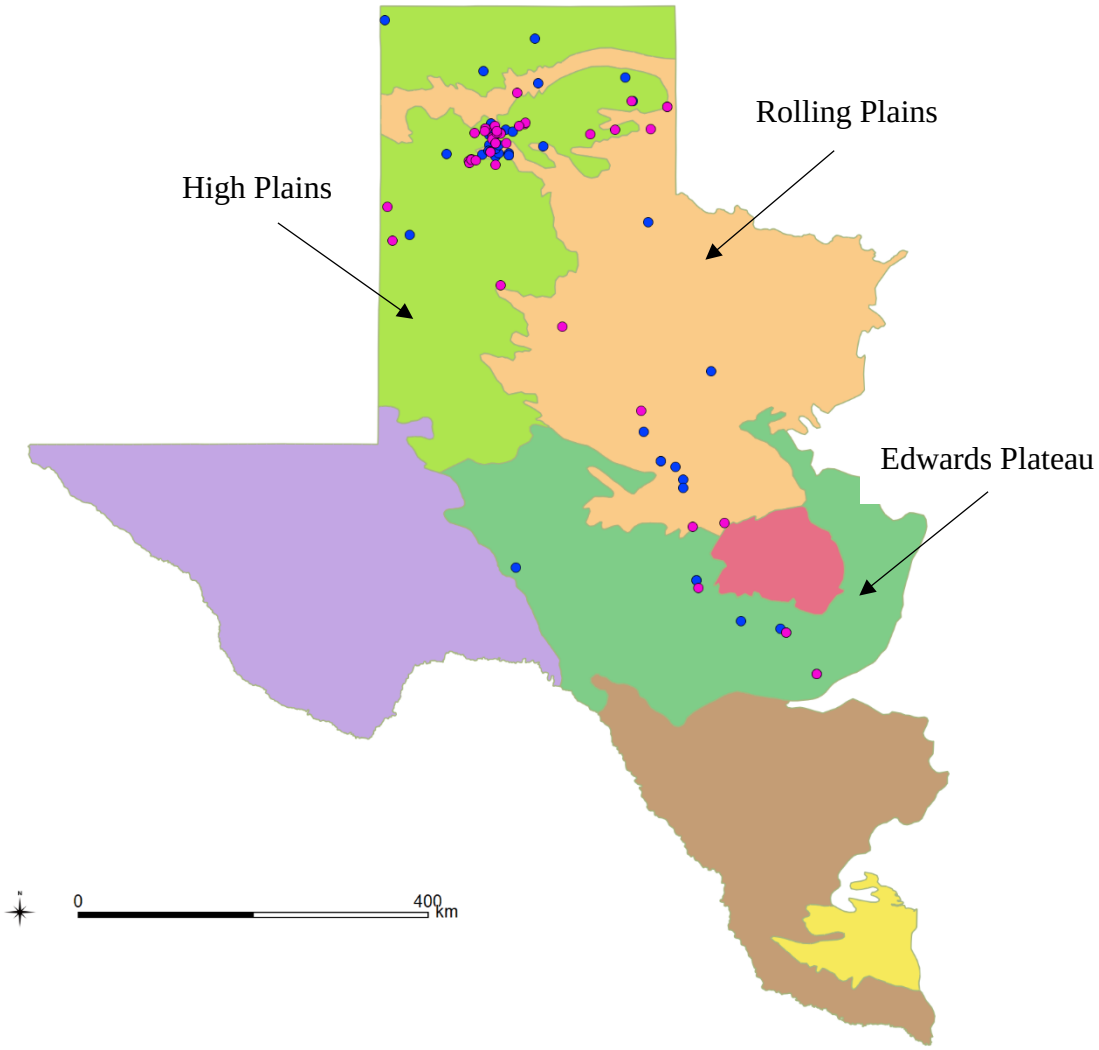

Figure II.1: GPS locations of 98 porcupine samples from across the High Plains, Rolling Plains, and Edwards Plateau ecoregions in western Texas. Males are denoted by blue circles and females are denoted by pink circles.

A) *Coendous*

Position (base pairs)

	38	43	50	58	80	88	106	120	121	124	133	142	148	155	163	166
Zfx	G	T	A	C	T	A	A	T	G	A	T	C	G	C	C	T
Zfy	T	G	G	T	C	T	G	C	A	T	C	T	A	A	G	A

B) *Erethizon*

Position (base pairs)

	31	43	49	58	80	88	106	120	121	124	133	142	148	155	163	166
Zfx	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T
Zfy	C	G	G	T	C	G	G	C	A	T	C	T	A	A	G	A

Table II.1: Sixteen variable positions (base pairs) of the sex-linked zinc finger protein gene in A) the prehensile tailed porcupine (*Coendous prehensilis*) (Woc-Colburn et al. 2013) and B) the North American porcupine (*Erethizon dorsatum*). Green columns indicate position variation concurrence between studies. Red columns indicate position variations specific to the data set of this study.

Table II.2: Ninety-two sequencing base pair profiles along 16 variable positions of the sex-linked zinc finger protein gene. Male-derived amplicons (n = 51) produced a heterozygous sequence (Zfx and Zfy) denoted by a ♂. Female-derived amplicons (n = 41) produced a homozygous sequence (Zfx only) denoted by a ♀. Ambiguous variation calls are denoted in orange and missing variation calls are denoted in yellow.

Position	**31**	**43**	**49**	**58**	**80**	**88**	**106**	**120**	**121**	**124**	**133**	**142**	**148**	**155**	**163**	**166**	
Zfx	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
Zfy	C	G	G	T	C	G	G	C	A	T	C	T	A	A	G	A	♂
ERDO_02	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_04	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_05	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_06	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_07	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_08	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_09	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_10	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_11	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_12	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_13	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_14	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_15	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_16	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_17	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_18	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_19	A	T	A	C	T	A	A	T	G	A	T						♀
ERDO_20	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_21	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_22	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀

ERDO_23	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_24	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_25	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_27	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_28	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_29	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_30	A	T	A	C	T	A	A	T	G	A	T	C					♀
ERDO_31	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_32	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_33	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_35	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_36	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C						♂
ERDO_37	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_38	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_39	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_40	A	T/G	A/G	C	T	A/G	A/G	T	G	A	T	C	G	T	C	T	♀
ERDO_41	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_42	A	T/G	A/G	C/T	T	A	A/G	T	G	A	T	C	G	T	C	T	♀
ERDO_45	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_46	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_47	A	T/G	A/G	C/T	T	A	A/G	T	G	A	T	C	G	T	C	T	♀
ERDO_48	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_49	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A			♂
ERDO_51	A	T?	A/G	C	T	A/G	A	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_52	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_53	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_54	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_55	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_56	A	T	A	C	T	A	A	T	G	A	T	C					♀
ERDO_57	A	T	A	C	T	A	A	T	G	A	T	C	G				♀
ERDO_58	A/C	T/G	A	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_59	A	T	A	C	T	A	A	T	G	A	T	C					♀

ERDO_60	A?	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A				♂
ERDO_61	A	T/G	A/G	C	T/C	A/G	A/G	T/C	G/A	A/T	T/C						♂
ERDO_63	A	T/G	A/G	C	T	A	A/G	T	G	A	T	C	G	T	C	T	♂
ERDO_64	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_66	A?	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_67	A?	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_68	A/C	T/G	A/G	C	T	A/G	A/G	T/C	G/A	A/T							♂
ERDO_69	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_70	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_71	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_72	A/C	T/G	A/G	C/T	T	A/G	A/G	T	G	A							♂
ERDO_73	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_74	A?	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_75	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_77		T	A	C				T	G	A	T						♀
ERDO_78	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_79	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_80	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_81	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_82	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_83	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_84	A?	T/G	A/G	C?	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_85	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_86	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T							♂
ERDO_88	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_89	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A			♂
ERDO_91	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_92	A	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_93	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_94	A?	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO_95	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO_96	A?	T/G	A/G	C/T	T?	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂

ERDO_99	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO100	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO102	A/C	T/G	A/G	C	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO103	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO104	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO105	A/C	T/G	A/G	C/T	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A	C/G	T/A	♂
ERDO106	A	T	A	C	T	A	A	T	G	A	T	C	G	T	C	T	♀
ERDO107	A?	T/G	A/G	C?	T/C	A/G	A/G	T/C	G/A	A/T	T/C	C/T	G/A	T/A			♂

Table II.3: Total number of male (♂) and female (♀) porcupines observed across 3 ecoregions during the 4-year sampling period. Sex was determined through a combination of generated sequencing data and anatomical evidence.

Ecoregion	**Males ♂**	**Females ♀**
High Plains	26	29
Rolling Plains	20	10
Edwards Plateau	7	6
Total	53	45