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An Initial Genetic Assessment of the Emblematic Pumas of the Torres del Paine UNESCO Biosphere Reserve

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Abstract: Physical and genetic isolation are recognized as significant threats to wildlife, especially in large carnivores inhabiting fragmented landscapes. We conducted an initial genetic assessment of pumas (*Puma concolor*) using 19 microsatellite loci for the emblematic puma population in the Torres del Paine UNESCO Biosphere Reserve in southernmost Chile, which exhibits some distinctive phenology that some local people speculate may be due to isolation and inbreeding depression. We extracted DNA from 385 scats collected in the field, of which 96 were identified as puma, representing 20 unique individuals. Torres del Paine pumas exhibited an H_o (0.51) only slightly lower than H_e (0.53), with 2 of the 19 loci significantly out of Hardy–Weinberg Equilibrium. Tests for a recent bottleneck of the population were not significant. The small sample size of individuals notwithstanding, these results seemingly do not support high levels of inbreeding. We also identified individual pumas in the field and assessed them for observable cowlicks (twirls of fur on their backs), a trait some have associated with genetic inbreeding depression in other puma populations. A total of 7 of 39 pumas exhibited cowlicks, consistent with geographic patterns of cowlicks within the species. Our tests exploring population structure among local pumas provided the most support for a single-population cluster, but we explored secondary structures as well, given its conservation implications. We encourage additional sampling in the region to further explore population structure and connectivity and determine the conservation status of the region's pumas to guide the development of best strategies to ensure their persistence.

Keywords: cowlick; genetics; microsatellite; *Puma concolor*; Torres del Paine

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1. Introduction

Physical and genetic isolation are now recognized as one of the most significant threats to wildlife, especially in large carnivores that inhabit landscapes fragmented by expanding human footprints [1]. Small populations isolated from others can exhibit lower allelic diversity and heterozygosity due to inbreeding and genetic drift, which in turn can lead to lower fitness (inbreeding depression) [2]. Inbreeding depression, defined as the homozygous state of deleterious, usually recessive alleles, often manifests phenotypically as a change in a physical trait, such as deformities, or a reduction in vital rates, such as survival or reproduction (e.g., [3,4]). As a result, diverse conservation strategies have been devised to facilitate an exchange of genetic material among populations, often called genetic rescue [5–7]. This conservation strategy involves introducing new genetic material

into an endangered or declining population to increase its genetic diversity and to reduce the effects of inbreeding on species fitness ([8] but see [9,10]).

Among large carnivores, pumas (*Puma concolor*), also called panthers, mountain lions, and cougars, are frequently studied to understand the consequences of isolation and inbreeding (e.g., Florida panthers [11,12], pumas in southern California [1,13]). Genetic patterns of puma populations across Latin America are less studied, even though they experienced similar patterns of range contraction and recovery as did pumas in North America (Figure 1) [14,15]. Extant pumas are thought to have evolved in South America [16,17], and the uniform pelage of adult pumas may in fact reflect an adaptation to open grassland habitats common in South America [18].

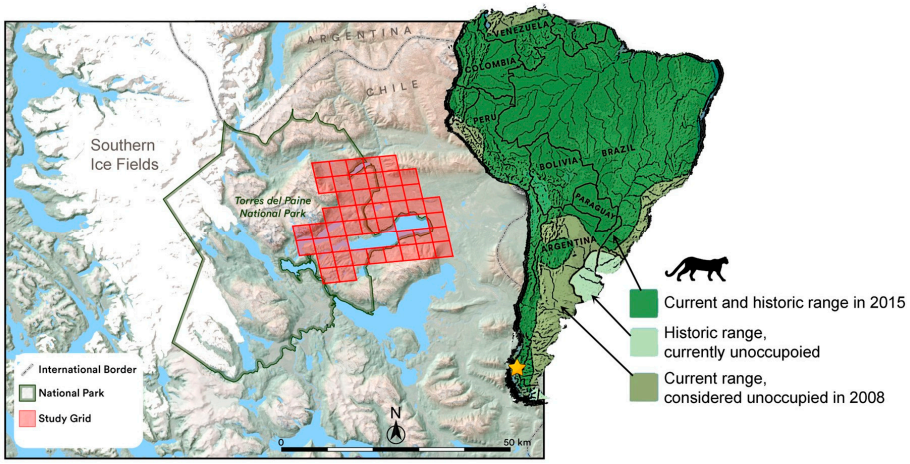

Figure 1. Study area and puma range. The main map on the left depicts the study area, highlighting the southern icefields to the west and north, the boundaries of Torres del Paine National Park, and the border between Chile and Argentina. The red squares are the 6 × 6 grid cells sampled for puma scats inside and adjacent to the National Park. On the right is a map of South America depicting a comparison of puma range published by the International Union for Conservation of Nature (IUCN) in 2008 and 2015, which may reflect recent recolonization of historic areas by pumas in South America. The study area's location is delineated by a yellow star.

The most observed puma population in the Americas inhabits the Torres del Paine UNESCO Biosphere Reserve (TdP) in southernmost Chile, where photographers, film makers, and wildlife enthusiasts flock to see pumas in the wild (e.g., the following films: BBC's *Dynasties II*, PBS's *Pumas: Legends of the Ice Mountains*). Many pumas from this population are easily differentiated from anywhere else in the puma's range due to distinctive facial characteristics—smaller eyes relative to their faces, narrower, squatter rostrums, and sometimes longer-than-typical ears, among other characteristics (Figure 2). This has led some local guides and biologists to speculate as to whether local phenotypes of TdP pumas are a result of historical microevolutionary processes such as mutation, drift, and genetic isolation supported by puma phylogeography [16], or whether they are indicative of more recent inbreeding and a potentially genetically compromised population. In other populations, for example, distinct facial features and swirls of fur (i.e., 'cowlicks') between the shoulders are suspected to be indicative characteristics of inbreeding (e.g., Florida panthers [11,12]) (Figure 2).

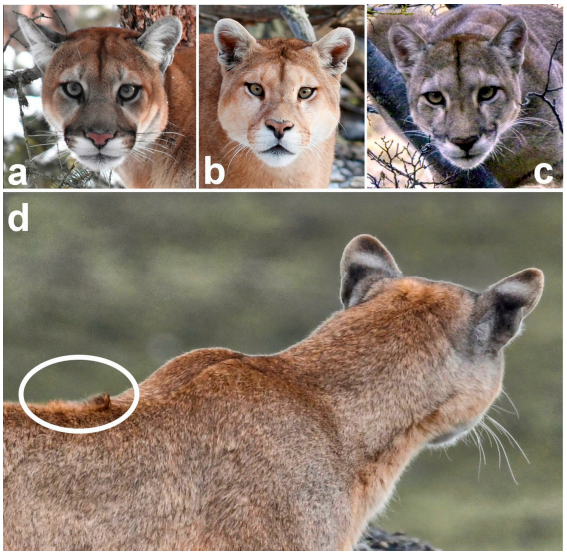

Figure 2. Puma phenotypes. Portraits of three female pumas, scaled so that their noses are equal in width, to emphasize differences in eye shape and size, rostrum shape and width, and the different shapes of their cheeks. Puma were from (**a**) Wyoming, (**b**) the Torres del Paine UNESCO Biosphere Preserve, and (**c**) Aysén, Chile, about 400 km north of Torres del Paine. In (**d**), we show a puma 'cowlick' photographed in the Torres del Paine UNESCO Biosphere Preserve during the study. Photographs (**a**,**c**,**d**) by Mark Elbroch and (**b**) by Nicolás Lagos.

Based upon the geography of the region and historic carnivore management practices, we hypothesized that the TdP puma population has experienced isolation, and perhaps genetic inbreeding, in the recent past. To the northwest of TdP lies the southern icefield, which is likely an absolute barrier to connectivity with puma populations to the north, and to the northeast and east lies Argentina, where ongoing provincial bounties and legal hunting [19,20] may result in a population sink or mortality rates that preclude the demographic conditions for successful dispersal and subsequent gene flow [14] (Figure 1). To the south in Chile, pumas are hunted, albeit illegally, despite the national protections in place since 1980 [21]. Killing pumas in southern Chile was, and may remain, socially acceptable, even among some wildlife agency staff [21]. Today, many Chilean ranchers in the region view Torres del Paine National Park as a sanctuary for pumas; in fact, ranchers further from the Park report seeing fewer pumas than those closer to the Park [22].

Here, we conducted an initial assessment of the phenotypic and genetic characteristics of pumas in the TdP that we hope prompts further conservation research to question whether local pumas have experienced isolation or genetic bottlenecks that might impact their phenotype or fitness. Such answers are important to supporting regional conservation strategies (e.g., Andean bear, *Tremarctos ornatus* [23]) and identifying core areas and critical corridors (e.g., jaguars, *Panthera onca* [24]). We hypothesized that pumas in the TdP population are a single, isolated population and that we would find low allelic diversity.

2. Materials and Methods

2.1. Study Area

The Torres del Paine UNESCO Biosphere Reserve encompasses 770,889 ha, inclusive of private lands and marine systems forming the buffer zone around Torres del Paine National Park. Our study area was the open steppe grassland habitat in the southern and eastern portions of Torres del Paine National Park (Permit RES.N°188) and the adjacent private lands of Estancia Laguna Amarga and Estancia Lazo in Chilean Patagonia (Figure 1). The landscape was Patagonia shrub-steppe, composed of xerophytic vegetation adapted to withstand drought and wind. Cushion shrubs and other short shrubs, including mata barrosa (*Mulinum spinosum*), calafate (*Berberis heterophylla*), mata guanaco (*Anarthrophyllum desideratum*), and mata negra (*Junellia tridens*), were common, with tufted grasses between

them. Precipitation occurs more commonly in winter from May to September and totals 100–300 mm annually [25]. Puma tourism has occurred in the area for 20 years but surged in growth beginning in 2014 in the National Park and on adjacent private lands [26].

2.2. Phenotypic Variation: Observable Cowlicks

We monitored pumas from 2017 to 2021 as part of tourism and wildlife filmmaking on the "Peninsula," an approximately 60–80 km^2 area of land north of Lake Sarmiento straddling the Torres del Paine National Park and adjacent private ranches. Individual pumas were identified using high-quality photos of their faces, distinctive markings, scars, and coloration [27]. We noted any observable cowlicks on pumas and reported that number as a percentage of total pumas observed.

2.3. Genetic Sampling

We divided our study system into 6×6 km quadrants (Figure 1), and in November 2019, we sampled each with a single transect walked by a trained scat-detecting dog (the handlers worked for Panthera and WorkingDogs4Conservation). Once scats were identified by dogs in the field, handlers recorded a GPS location and placed a 2-inch segment in a container with desiccant to dry the sample for storage and transport.

In 2020, 385 scat samples were shipped to the USDA Forest Service's National Genomics Center for Wildlife and Fish Conservation in Missoula, MT, USA, for genetic analyses. DNA was extracted using the QIAGEN Qiamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To determine the species, we first used the 16s rRNA region [28] of mitochondrial DNA and subsequently a small portion of the cytochrome b gene for samples that initially failed [29]. All sequences were checked by eye and aligned with Sequencher v.5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA). Any ambiguous nucleotide calls were recorded as missing data. The resulting sequence was compared to reference sequences via BLAST search in the nucleotide database on GenBank to confirm the species based on the "percent identity" field in the BLAST results. Samples clearly identified as puma (typically in the high 90th percentile) were analyzed for individual identification using a panel of 19 microsatellite loci known to be variable for pumas: Fca08, Fca35, Fca43, Fca57, Fca77, Fca82, Fca90, Fca96, Fca132, Fca149, Fca176, Fca391, Fca559, LC109 [16,30], PcoA208, PcoB010, PcoB210, PcoC108, and PcoC112 [31]. DNA sex identification was performed using felid-specific sexing markers that detect deletions in the zinc-finger and amelogenin regions of the Y-chromosome [32].

Quality control of the genotypes obtained from fecal samples is of utmost importance. To ensure the rigor of our dataset, we employed a number of checks. All alleles in the dataset observed at low frequency (<0.05) were re-amplified. The entire dataset was then analyzed using the computer screening programs DROPOUT [33], MICRO-CHECKER 2.2.3 [34], and GenAlEx 6.5 [35]. We used the program MICRO-CHECKER 2.2.3 to check for null alleles. We performed replicate genotyping in which each sample was PCR-amplified twice across the 19 variable microsatellite loci to screen for allele dropout, stutter artifacts, and false alleles [36]. If any locus failed to amplify in either replicate or if there was a discrepancy in PCR amplification, the sample was repeated twice more to minimize genotyping error. If a genotype was confirmed by this repeat analysis, then it was retained. If a genotype failed again, the sample was assigned a missing score at the failed locus.

We removed any sample for which amplification failed at ≥ one-third of loci and screened remaining genotypes to detect and correct genotyping error using program DROPOUT 2.3 and the DCH test [33,37]. This test uses a subset of loci that achieve a reasonable probability of individual identity and then tests what occurs when an additional new locus is added to this subset. Once the minimum number of loci needed to identify unique individuals is achieved, any additional locus should not add a new individual. If a new individual is detected when a new locus is added, then it is probably due to a genotyping error at that locus. In this dataset, no microsatellite locus was found to have errors falsely increasing the number of individuals.

2.4. Genetic Diversity and Inbreeding

We calculated standard estimates of genetic diversity for our TdP pumas using GenAlEx. These included observed, expected, and unbiased expected heterozygosity (H_o, H_e, and uH_e, respectively), allele number (N_a) and effective number of alleles (N_e), and the inbreeding coefficient F_{IS} (a measure of departure from Hardy–Weinberg proportions). We also used GenAlEx 6.5 to test whether each locus was in an expected Hardy–Weinberg proportion and the program GenePop 4.7 [38] to check for linkage disequilibrium.

We assessed this population for evidence of a recent reduction in effective population size using the program BOTTLENECK [39]. Populations that have experienced a recent genetic bottleneck exhibit a correlative reduction in allele numbers and heterozygosity at polymorphic loci. However, allelic numbers are reduced faster than gene diversity. Thus, a recently reduced population is characterized when observed heterozygosity is larger than expected equilibrium heterozygosity, which is calculated from the observed number of alleles under the assumption of a constant-size population (mutation–drift equilibrium) [40]. Most microsatellite datasets fit a two-phase model of mutation (TPM) rather than the infinite allele model (IAM) or stepwise mutation model (SMM) [41]. Therefore, our analysis was conducted using a TPM with multistep mutations, accounting for 5%, 10%, 20%, or 30% of all mutations. A Wilcoxon signed-rank test was used to determine which populations had a significant number of loci with gene diversity excess [42].

2.5. Population Structure

We employed a Bayesian clustering analysis in program STRUCTURE v2.3.4 [43] to determine the probability of individual puma assignment among 1-5 independent clusters (K = 1–5). We assessed all values of K with 5 replicates, each beginning with a burn-in period of 100,000 runs, followed by 1,000,000 iterations. We used an admixture model, correlated allele frequencies, and excluded sampling location priors. Using the program STRUCTURE HARVESTER [44], we assessed the mean and potentially overlapping standard deviations of the posterior probability (L(K)) as support for any one or more K [43]. We did not use ΔK [45], as that ad hoc criterion cannot find the best K if K = 1. Additionally, to further determine whether individual pumas represented a distinctive population structure, we conducted a principal coordinate analysis (PCoA). We used the software GenAlEx 6.5 [35], selecting the option for a covariance matrix from standardized data calculated from all microsatellite loci. PCoA graphically represents the genetic similarity among individuals based on their pairwise genetic distances.

3. Results

3.1. Cowlicks

From 2017 to 2021, we observed 39 individual pumas in the area, of which 7 (18%) had dorsal cowlicks (Figure 2). These included three males and four females.

3.2. Samples and Population Structure

We gathered 385 scats in the field, 96 of which were identified as puma via the mtDNA 16s and cytochrome *b* sequences (Table S1). The majority, n = 146, were scats of South American gray fox (*Lycalopex griseus*), and a large number of samples (n = 128) did not adequately amplify for species ID. The remainder of the 385 scats were domestic dog (n = 2), Geoffroy's cat (n = 6; *Leopardus geoffroyi*), and culpeo fox (n = 7; *Lycalopex culpaeus*). Of the 96 puma scats, many had DNA quality too poor to pass minimal criteria in our quality control steps to provide for individual or sex identification. In total, we confirmed sex ID in 38 puma samples (40.0%) and individual identification from 23 puma samples (24%). Direct-count repeat genotyping error rates per allele ranged from 0.00 to 0.027 across samples and loci. We identified 20 unique pumas: 13 females and 7 males (Table S2).

We estimated the probability of identity (PID [46]), the probability that two individuals sampled at random will have the same genotype, and the probability that siblings are identical (PIDsib [47]) for these data to demonstrate adequate statistical power for

discriminating unique individuals. Calculations for PID and PIDsib were 2.001×10^{-12} and 8.16×10^{-6}, respectively. Values supported the high discriminatory power of our markers [48]. Overall, the samples exhibited H_o = 0.511 (0.056 SE), H_e = 0.534 (0.047 SE), and uH_e = 0.55 (0.049 SE). The number of effective alleles (N_e) was less than the number of alleles (N_a) at 2.577 and 4.211, respectively, indicating that allele frequencies per locus were uneven. The fixation index, F_{IS} = 0.044 (SE 0.055), indicated no excess of homozygotes or heterozygotes. After correcting for multiple tests (Bonferroni correction), two loci (FCA96 and FCA559) were not in Hardy–Weinberg proportion (Table S3) and were removed in analyses sensitive to HWP. After correcting for multiple tests, linkage disequilibrium was not significant in any pair of loci.

Wilcoxon tests of significance were consistent across the IAM, SMM, and four TPM scenarios used. After correcting for multiple tests, significant excess heterozygosity (one-tailed Wilcoxon test for H excess) was not detected in any scenario for this population (all $p > 0.11$).

Bayesian clustering analysis showed the greatest support for the sampled pumas composing a single population (Figure S1; K = 1). In exploring the patterns of other values of K, there appeared to be a putative secondary structure at K = 2 (Table S4). The PCoA of the 20 individuals showed that 14.99% and 13.04% of the variation (28.03% total) could be explained by coordinates 1 and 2, respectively. When the same individuals split at K = 2 are plotted with the first two coordinates of the PCoA, they roughly segregate into two groups, mostly along coordinate 1 (Figure 3).

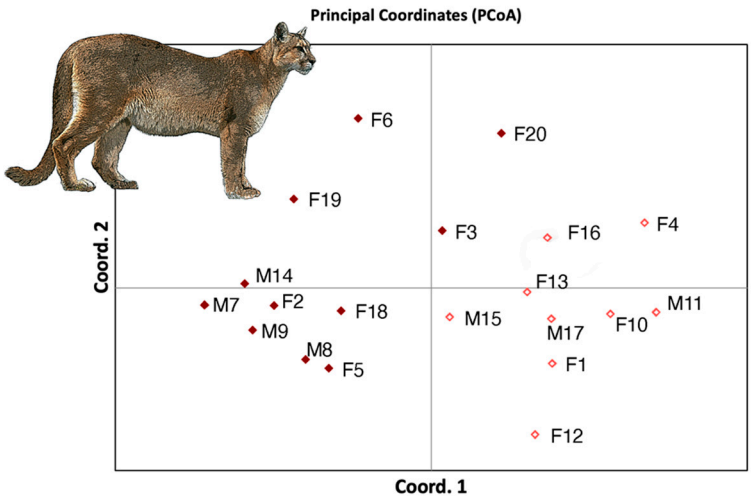

Figure 3. Principle coordinate analysis (PCoA) of 20 individual pumas, labeled by alternative IDs (Table S4) denoting males (M) and females (F). Figure shows individual locations relative to the first two components of the PCoA of allele frequencies at 19 microsatellite loci. Dark, solid diamonds versus light diamond outlines indicates >0.50 assignment to one of two clusters per the STRUCTURE analysis at K = 2 (Table S4).

4. Discussion

The pumas of the Torres del Paine UNESCO Biosphere Reserve exhibited moderate levels of genetic diversity among nuclear microsatellites and showed no indication of a genetic bottleneck. Of the pumas observed in the wild, 18% had dorsal cowlicks (also called whorls), that may or may not be indicative of inbreeding [11,12]. Wilkens et al. [49] assessed presence of cowlicks in 96 live pumas from North America as well as 648 puma skins from museum collections across North and South America. They found a frequency of occurrence of cowlicks in 79.2% of Florida panthers (n = 72) but only in 1.75% of North American pumas outside of Florida (n = 456). Interestingly, they tabulated a 14.4% prevalence in South America (n = 145), with an even higher rate of 27% in pumas from Chile and Argentina (n = 74). Thus, the prevalence detected in TdP (18%) is within the range expected from this examination of archived data [49]. This does not preclude the possibility that samples from Wilkins et al. [49] were or were not from a population, or populations, affected by

inbreeding, but the more plausible conclusion is that cowlicks are more frequently found in South American puma populations, and this prevalence is not an indicator of inbreeding depression. In aggregate, we did not find evidence to suggest that the TdP pumas require translocations or genetic rescue to improve genetic diversity.

In support of our hypotheses, the best evidence suggested that the individuals we sampled represented a single population cluster, but we lacked the data needed to determine whether this cluster matched genetic characteristics of pumas in nearby Argentina or further from TdP. There have been genetic analyses completed for several other puma populations in South America (e.g., $H_o = 0.63$, $H_e = 0.73$ in central Argentina [14]; $H_o = 0.609$ in southeast Brazil [50]), but unfortunately, due to using different loci in our analyses, we could not directly compare our diversity metrics, nor were we able to use these studies to assess levels of connectivity among puma populations.

Our PCoA analysis showed a segregation of individuals along PCoA coordinate 1 (Figure 3) that matches the uneven (i.e., >50%) assignment of individuals to one of two groups in the STRUCTURE results of $K = 2$ (Table S4), although these were not our most supported results. This may indicate that individuals sampled from this population have ancestry derived from two genetically distinguishable populations, or it may only represent fine-scale, stochastic differences expected to randomly emerge given these analytical approaches.

Pumas have begun repopulating the grasslands of Argentina in the last decade [15,51], and one hypothesis drawn from our non-significant STRUCTURE results for $K = 2$ is that we may be witnessing admixture due to recent immigration into the Torres del Paine UNESCO Biosphere Reserve arriving via Argentina or south of TdP (*sensu* [52]). Recent immigrants into TdP could explain the strong heterozygosity (H_o, H_e) values we calculated, as research in North America has shown the benefits of just a single migrant to an isolated population [1].

5. Conclusions

We began with the assumption that the pumas in our study system would show the genetic characteristics representative of an isolated population due to a barrier effect of the southern ice fields to the north and west and the ongoing aggressive provincial management of pumas in Argentina to the east. Our results, however, support a genetically healthy and viable population. This may be due to a larger-than-expected local population or that there is in fact sufficient gene flow with a neighboring population(s) either via reciprocal exchange of migrants or by new individuals emigrating from nearby expanding populations.

While our STRUCTURE analysis clearly showed statistical support for sampled individuals forming a single population, patterns of assignment around alternative values of K can and should be used as an exploratory tool to devise new hypotheses [43,53,54]. For the TdP pumas, the STRUCTURE and PCoA results (Figure 3) provide potentially interesting patterns of substructure around $K = 2$. Both analyses revealed individual-based patterns of genetic diversity that might be expected if the pumas sampled had ancestry from two distinctive populations. If our results indicate admixture, then Torres del Paine represents a contact zone where distinctive populations are mixing following the period when puma persecution in open grasslands was highest (Novaro and Walker 2021). There is also the possibility that our results are an artifact of limited sampling distribution and a small sample size [54].

Culver et al. [16] suggested that pumas ranging the full length of Chile and much of Argentina represent a single subspecies, reflecting significant population connectivity. However, more data are required to better determine whether the pumas of the Torres del Paine UNESCO Biosphere Reserve have become isolated from other populations in the region and their relative genetic health. To test new hypotheses around the admixture of genetically diverse historical puma populations, additional genetic sampling is needed, including from individuals collected more broadly across all of Patagonia, from different populations. Future work could also include assessing a reduced-representation panel of

SNPs (e.g., [55]) or the full genome and evaluating runs of homozygosity (ROH) [17], which can reflect historic impacts of inbreeding depression even after more recent admixture has improved heterozygosity. This is an important question in determining the conservation status of the region's pumas and developing the best strategies to ensure their persistence.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16090581/s1, Table S1: Species results for 385 scats; Table S2: Pumas in PCoA and structure; Table S3: Per locus descriptive statistics analyses; Table S4: Results of STRUCTURE with $K = 2$; Figure S1: Bayesian analysis comparing $K = 1$ to 5.

Author Contributions: Conceptualization, L.M.E., B.V.W. and O.O.; methodology, B.V.W., K.P. and M.K.S.; software, B.V.W.; validation, B.V.W., K.P. and M.K.S.; formal analysis, B.V.W.; investigation, L.M.E., O.O., N.L., S.A.-A., M.M. and D.G.; resources, B.V.W.; data curation, K.P. and S.A.-A.; writing—original draft preparation, L.M.E. and B.V.W.; writing—review and editing, L.M.E., B.V.W., K.P., O.O., N.L., S.A.-A., M.M., D.G. and M.K.S.; visualization, L.M.E., B.V.W. and M.K.S.; supervision, L.M.E. and M.K.S.; project administration, L.M.E.; funding acquisition, L.M.E. All authors have read and agreed to the published version of the manuscript.

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