Undergraduate Thesis

Potential Hybridization Among Three Colorado Cottontail Rabbit Species

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Abstract

Humans are modifying the environment and altering the habitable ranges of species, bringing together species that were previously geographically separated and providing new opportunities for hybridization. Determining how range expansion and urbanization have impacted species interactions will allow us to better understand the influence humans are having on speciation and hybridization. I focused on three cottontail rabbit species (eastern, desert, and mountain; genus *Sylvilagus*) with human-disrupted ranges that currently converge along the Front Range Urban Corridor. It is unknown what impact human disturbance has had on these species and whether it has led to possible hybridization. Using museum specimens, I developed a method to genetically identify the species of each cottontail rabbit by sequencing the *cytb* mitochondrial gene. To assess if the three species are hybridizing, I then compared the species identity determined by mitochondrial genetics to the species identity indicated by morphology, with disagreement between the two indicating possible hybridization. I found three individuals that show signatures consistent with hybridization, but additional sequencing is needed for confirmation. Hybridization could lead to a breakdown in mating barriers and may redefine our definitions of these three cottontail species. If the species aren't hybridizing, further research should be done to understand how mating barriers are being maintained.

Introduction

Humans have long been altering the environment around them, from farming and urban development to pollution and climate change. It is important to understand human influence on environments and the species that live within them to better understand human impact on the Earth (Grabenstein et al. 2018). As humans spread across the western United States, they altered the landscape through development and urbanization, and previously geographically isolated species were provided the opportunity for range expansion into overlapping habitats (Taylor et al. 2015). Once in contact, individuals of two distinct species can hybridize, or mate and produce viable offspring (Barton et al. 1989). An outstanding question is are the new overlapping habitats provided by human disturbance leading to hybridization among once isolated lineages and what effect are humans having on evolutionary processes, like speciation and hybridization?

There are two possible outcomes of hybridization. Species that were once distinct can fuse together into a single species (*e.g.* Hasselman et al. 2014). Alternatively, species may interbreed but not extensively, or their offspring may be sterile, which further prevents gene flow despite interbreeding (*e.g.* Taylor et al. 2014). In either case, hybridization is an opportunity to study how species interact. To answer the question of whether hybridization is occurring, it is necessary to look for gene flow between distinct lineages of species. Looking for gene flow requires the genetic sequencing of individuals found where species ranges overlap and have the opportunity to interbreed. Within the genotype of the individual, we determine if introgression, where alleles from one species are incorporated into another, is occurring (Gompert et al. 2017). If the species have remained distinct, we expect to find most individuals with genotypes similar to their parents, and few individuals that have alleles from both species (Harrison and Larson 2014). If the species have fused, we expect to find extensive admixture, or incorporation of alleles from both parents into the genome, making these populations essentially indistinguishable (Hasselman et al. 2014). It is also possible for species to fall somewhere in between, where the only evidence of past hybridization is introgression of the maternally inherited mitochondrial DNA (mtDNA). The introgression of maternal mtDNA is referred to as ‘mitochondrial capture’ and is common in mammals that rarely hybridize such as North American chipmunks (Good et al. 2008).

Here, I will ask if hybridization is occurring among three species of North American cottontail rabbits, the desert cottontail (*Sylvilagus audubonii*), the mountain cottontail (*S.*

nuttallii), and the eastern cottontail (*S. floridanus*), whose ranges overlap in the Front Range Urban Corridor of Colorado. The desert cottontail can be found from Mexico to the northern border of the United States and the mountain cottontail can be found from New Mexico to the northern border of the United States and into Canada. Historically, these two species were the only cottontails that occupied Colorado habitats, but lived at different elevations (Smith et al. 2018). Likely due to increased habitat availability through urbanization, the mountain and desert cottontails can now both be found at lower elevations. Likewise, the eastern cottontail was formerly limited to the eastern United States, but this generalist species has recently expanded its range and can now be found in the Rocky Mountain West, including Denver (Nielsen and Lanier, 2019). These three species' ranges now overlap in urban and suburban areas along the Colorado Front Range, allowing for hybridization to potentially occur. Hybridization among these three species may be likely given that eastern cottontails have hybridized with other species within their spreading habitat range (Verts and Carraway 1980) and hybridization appears to be common among species within the lagomorph order, such as snowshoe hares and jackrabbits (Jones et al. 2018) and European rabbits (Carneiro et al. 2013).

It is currently unknown what impact human disturbance has had on these species and whether it has led to possible hybridization. Little research has been conducted on the influence of human urbanization on cottontail species interactions and their possible hybridization. I hypothesize that the impacts of human disturbance and expanding species ranges will allow hybridization to occur among Colorado cottontail species. First, I expect eastern cottontails to hybridize with both desert and mountain cottontails. Eastern cottontails are successful invaders of new territory, have been found to have hybridized with other rabbit species when they do invade (Verts and Carraway, 1980), and have more aggressive mating behaviors than desert and mountain cottontails (Davis and Roth, 2007). Second, I expect desert and mountain cottontails to not interbreed, despite their now overlapping ranges, because these two species have had a longer history of occupying similar environments and may be more likely to have evolved reproductive barriers to interbreeding (Halanych and Robinson, 1996). Here, I use mtDNA sequencing of cottontail individuals from each species across the Front Range to test if hybridization and introgression are occurring and to what extent. The results of this study will provide insight into how range expansions and urbanization influence species boundaries. If hybridization is occurring, it could suggest that mating barriers have been broken down or never

existed in the first place, leading us to redefine our delineation of these three cottontail species. If the species aren't hybridizing, further research should be done to understand how mating barriers are being maintained.

Methods

I conducted genetic analysis on three cottontail species: the eastern, desert, and mountain cottontails, using samples from across the Front Range Urban Corridor to look for hybridization (see Figure 1). Dr. John Demboski from the Denver Museum of Nature and Science (DMNS) provided tissue samples. The museum staff at DMNS determined the species of each rabbit sample using morphology, though the three species can be difficult to identify, meaning some were identified as "unknown." A total of 28 samples were sequenced, with 11 morphologically identified as *S. audubonii*, nine identified as *S. floridanus*, one identified as *S. nuttallii*, and seven had an unknown identification (see Table 1).

DNA extractions were performed by K. Hunnicutt. Briefly, DNA was extracted from liver, heart, or muscle samples from DMNS specimens using the NucleoSpin Tissue kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocol. The extractions yielded rabbit DNA at sufficient concentrations for polymerase chain reaction (PCR) amplification. DNA was quantified with QuantiFluor dsDNA System fluorescent dye (Promega) and the Synergy HTX Multi-Mode Microplate Reader (Agilent).

To evaluate species identity of both morphologically defined and unidentified cottontail samples, I sequenced each sample's mtDNA. I chose to sequence mtDNA over nuclear DNA as it evolves more rapidly and does not recombine. It is also haploid, meaning only one copy is present rather than two, making it easier to create a consensus sequence without multiple alleles, insertions, deletions, or single nucleotide polymorphisms (SNPs) (Bowles et al. 1992). To decide which segment of the mitochondria would be most appropriate for determining if hybridization is occurring, I performed an initial PCR amplification of three mitochondrial regions, *12S rRNA*, the *d-loop* region and the *tRNA-Thr* and *tRNA-Pro* genes (collectively *d-loop* hereafter), and *cytochrome-b* (*cytb*), on one individual from each species (see Table 2 for primers).

Following PCR amplification, I used gel electrophoresis to confirm that the PCR amplification was successful and uncontaminated, both in product length and concentration. Then to prepare the samples for sequencing, I purified the PCR products using ExoSAP-IT

Express (ThermoFisher Scientific, Waltham, Massachusetts) with a modified protocol that diluted the reagent in a 1:1 ratio with water to remove remaining reagents and unused primers. I used Sanger sequencing to sequence the amplified mitochondrial genes with GENEWIZ (Brooks Life Sciences, South Plainfield, New Jersey). Chromatograms were first edited manually in Geneious Prime v. 2021.1.1 (Geneious, Auckland, New Zealand). For each region, I aligned the cleaned sequences from each target species (three samples) to reference previously sequenced *Sylvilagus* and pika sequences downloaded from NCBI GenBank (110 samples) using MAFFT v. 7.450 (Research Institute for Microbial Diseases, Osaka, Japan). Then I used each alignment to build a phylogenetic tree using RAxML v. 8.2.11 (The Exelixis Lab, Heidelberg, Germany) with the GTR GAMMA I nucleotide model and Rapid Bootstrapping with search for the best-scoring maximum likelihood tree algorithm with 10000 bootstrap replicates. I compared the three gene segments for the initial three individuals to determine which mitochondrial gene was the most appropriate for analysis. Several conditions were considered that made *cytb* the most appropriate gene for further analysis. *Cytb* sequencing produced the longest cleaned sequencing reads, provided the clearest sorting of the three focal species into separate clades, and had the most available reference sequences of each of the three target species. Additionally, *cytb* is the only coding region of the three genes, making alignment easier. As coding regions of the genome are under more selection pressure, there is less variation among reads and more conservation across species. While cleaning reads, we can also rule out mutations that cause premature stop codons that would lead to a non-functional copy of this gene.

After choosing the *cytb* sequence for analysis of hybridization, I repeated the PCR amplification process to sequence an additional 25 individuals for a total of 28 *cytb* samples. I then cleaned and aligned the sequences again using Geneious software. Following the same alignment and tree building protocol above, I built a phylogenetic tree with additional reference sequences to determine the genetic identification of each individual based on the clade into which it was sorted. Reference sequences from the three focal species, as well as other closely related species and two outgroups, the American and collared pikas, were used. I then compared the genetic identification of each individual with its morphological identity determined by museum staff. Identifying a mismatch could mean the introgression of alleles from another species, thus indicating a hybrid individual, or indicate that the morphological identification of the individual was incorrect.

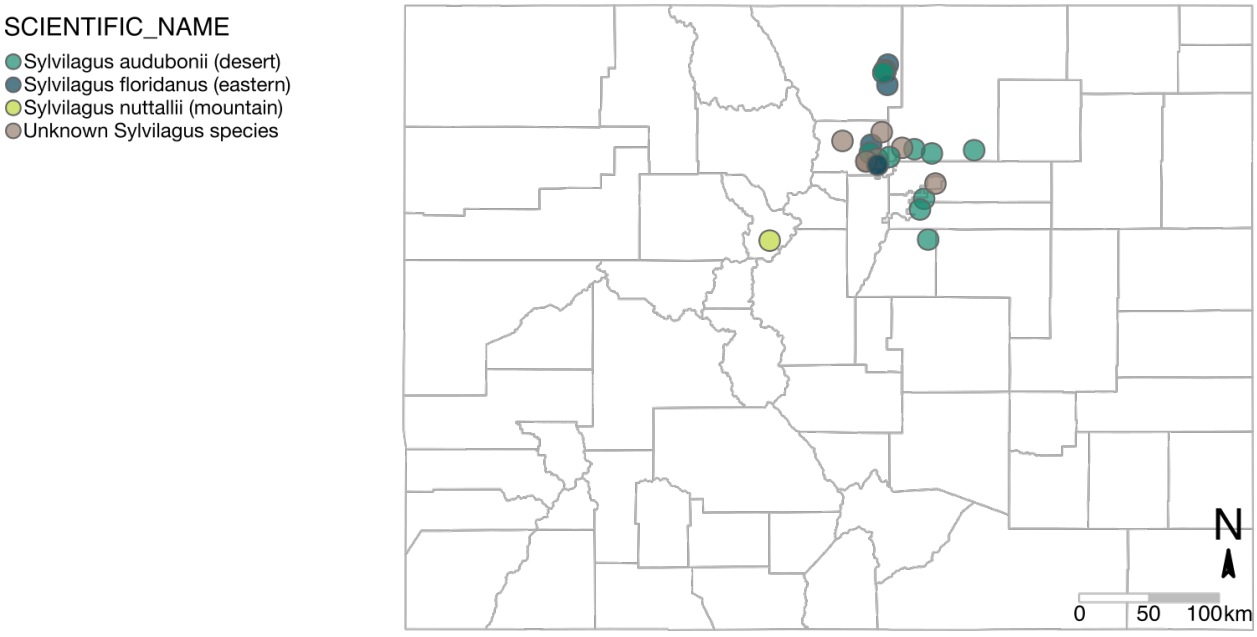

Figure 1. Map of Colorado indicating location of each cottontail sample, color coded by species identification. Figure by K. Hunnicutt.

Table 1. Morphological and genetic identities and geographical distributions of the samples included in this study. Individuals labelled as *Sylvilagus sp.* were of unknown morphological ID. Individuals that have different morphological and genetic identity are indicated by gray shading. NA indicates elevation information was not available for this individual.

Sample ID	Sex	Morph ID	Genetic ID	Elevation	Latitude	Longitude
DZTM 6114	female	*S. audubonii*	*S. audubonii*	1525-1525	40.6	-105.1
DZTM 6115	female	*S. audubonii*	*S. audubonii*	1525-1525	40.6	-105.1
DZTM 6121	male	*S. audubonii*	*S. audubonii*	1496-1496	40.1	-104.8
DZTM 6215	male	*S. audubonii*	*S. audubonii*	1538-1538	40.6	-105.1
DZTM 6272	female	*Sylvilagus sp.*	*S. audubonii*	1532-1532	40.1	-104.9
DZTM 6731	unknown	*Sylvilagus sp.*	*S. audubonii*	1536-1536	40.2	-105.1
DZTM 6732	male	*S. audubonii*	*S. audubonii*	1478-1478	40.1	-104.3
DZTM 6740	unknown	*S. floridanus*	*S. floridanus*	1705-1705	40.0	-105.2
DZTM 6752	male	*S. nuttallii*	*S. floridanus*	NA	39.5	-106.0
DZTM 6755	male	*S. audubonii*	*S. audubonii*	1883-1883	39.5	-104.7
DZTM 6759	female	*S. audubonii*	*S. audubonii*	1525-1525	40.1	-104.7
DZTM 6766	male	*S. audubonii*	*S. audubonii*	NA	40.1	-105.2
DZTM 6776	unknown	*Sylvilagus sp.*	*S. floridanus*	2605-2605	40.1	-105.4
DZTM 6785	unknown	*S. audubonii*	*S. audubonii*	NA	40.0	-105.0
DZTM 6789	female	*Sylvilagus sp.*	*S. audubonii*	1600-1650	39.9	-104.7
DZTM 6791	female	*S. audubonii*	*S. audubonii*	NA	39.8	-104.8
DZTM 6805	male	*S. audubonii*	*S. audubonii*	1699-1699	39.7	-104.8
DZTM 6818	unknown	*Sylvilagus sp.*	*S. audubonii*	1690-1690	40.0	-105.1
DZTM 6857	female	*Sylvilagus sp.*	*S. audubonii*	1617-1617	40.0	-105.2
DZTM 6858	female	*Sylvilagus sp.*	*S. audubonii*	1617-1617	40.0	-105.2
DZTM 7287	male	*S. floridanus*	*S. audubonii*	1523-1523	40.5	-105.1
DZTM 7291	male	*S. floridanus*	*S. floridanus*	1548-1548	40.6	-105.1
DZTM 7295	male	*S. floridanus*	*S. floridanus*	1570-1570	40.1	-105.2
DZTM 7330	female	*S. floridanus*	*S. audubonii*	1659-1659	40.0	-105.1
DZTM 7335	female	*S. floridanus*	*S. floridanus*	NA	40.6	-105.1
DZTM 7336	unknown	*S. floridanus*	*S. floridanus*	NA	40.6	-105.1
DZTM 7337	unknown	*S. floridanus*	*S. floridanus*	NA	40.6	-105.1
DZTM 7338	male	*S. floridanus*	*S. floridanus*	NA	40.6	-105.1

Table 2. Primers used in this study and amplification conditions for each primer set. Primers indicated with an asterisk (*) were modified by K. Hunnicutt to match the *Oryctolagus cuniculus* reference mitochondrial genome (NCBI; Accession number NC_001913).

Name	Sequence (5' to 3')	Melting Temp. (°C)	Annealing Temp. (°C)	Product Length (bases)	Region	Original publication
CytB_L14724a_OCun_F	TGA CTA ATG ACA TGA AAA ATC ATC GTT	62	61	~1000	*cytb*	*modified from (Irwin et al. 1991)
CytB_new_OCun_R	TTA ATC TCC GTT TCT GGT TTA CAA GAC C	65	61	~400 - 1000	*cytb*	*modified from (Yoder et al. 2016)
CyB_Lemur_R	TCT CCA TTT CTG GTT TAC AAG ACC A	63	61	~1000	*cytb*	Yoder et al. 2016
Sylv_CytB_Int_R1	ACT GGG RCC TTC ATT TGA GG	56	61	~1000	Internal *cytb*	*This study
12S_A_OCun_F	CAT AAA CAT AAA GGT TTG GTC C	55	61	~700	*12S rRNA*	*modified from (Allard and Honeycutt, 1992)
12S_D_OCun_R	CAC TTG AGG AGG GTG ACG GGC GGT GTG T	79	61	~700	*12S rRNA*	*modified from (Allard and Honeycutt, 1992)
Dloop_L15934_OCun_F	CTC TGG TCT TGT AAG CCA GGA ATG G	66	61	~550	*d-loop region, tRNA-Thr* and *tRNA-Pro*	*modified from (Litvaitis et al. 1997)
Dloop_H16498_OCun_R	CCT GAG GTA GTA AGA ACC AGA TG	55	61	~550	*d-loop region, tRNA-Thr,* and *tRNA-Pro*	*modified from (Meyer et al. 1990)

Results

For each of the three mitochondria regions I suspected to be good candidates for studying hybridization in Colorado cottontail rabbits, I sequenced three individuals, a *S. floridanus*, a *S. nuttallii*, and a *S. audubonii*. Using these sequences, I built a phylogenetic tree to see how well the test individuals for each species sorted into clades. Based on these parameters, I determined the *cytb* gene to be the most useful for study. In total, 28 samples were successfully amplified for the focal gene, *cytb*, which yielded a product with a length of approximately 1,150 bases. Approximately 80 single SNPs were used to align the 28 *cytb* sequences with reference sequences from the National Center for Biotechnology Information (NCBI) Genbank. The alignment was then used to build a final phylogenetic tree representing all *cytb* samples and reference sequences (Figures 2 and 3).

The phylogenetic trees below represent the grouping into clades of each *Sylvilagus* sample plus an additional 95 reference sequences covering nine *Sylvilagus* species from across North America, Central America, and northern South America (Figures 2 and 3). These nine species comprised eight major clades. Consistent with previous phylogenetic trees of these lineages, the desert and mountain cottontails formed a separate clade from eastern cottontails, indicating that desert and mountain cottontails are more closely related and are likely sister species (Halanych and Robinson, 1996). Eastern cottontails formed a single clade with another species of cottontails, the Davis Mountains (robust) cottontail.

For 25 sequenced individuals, the genetic species identification matched the morphological species identification (Table 1). Seven morphologically ambiguous individuals who were previously unidentified were able to be identified through mtDNA sequencing. The majority of unknown cottontails group with the desert cottontail, with a single unknown cottontail grouping with the eastern cottontail.

Three sequenced individuals did not sort with their morphological clade, meaning their genetic species identification does not match their morphological species clade. Two of these individuals, one male and one female, were morphologically identified as eastern cottontails and genetically identified as desert cottontails. One male individual was morphologically identified as a mountain cottontail and genetically identified as an eastern cottontail.

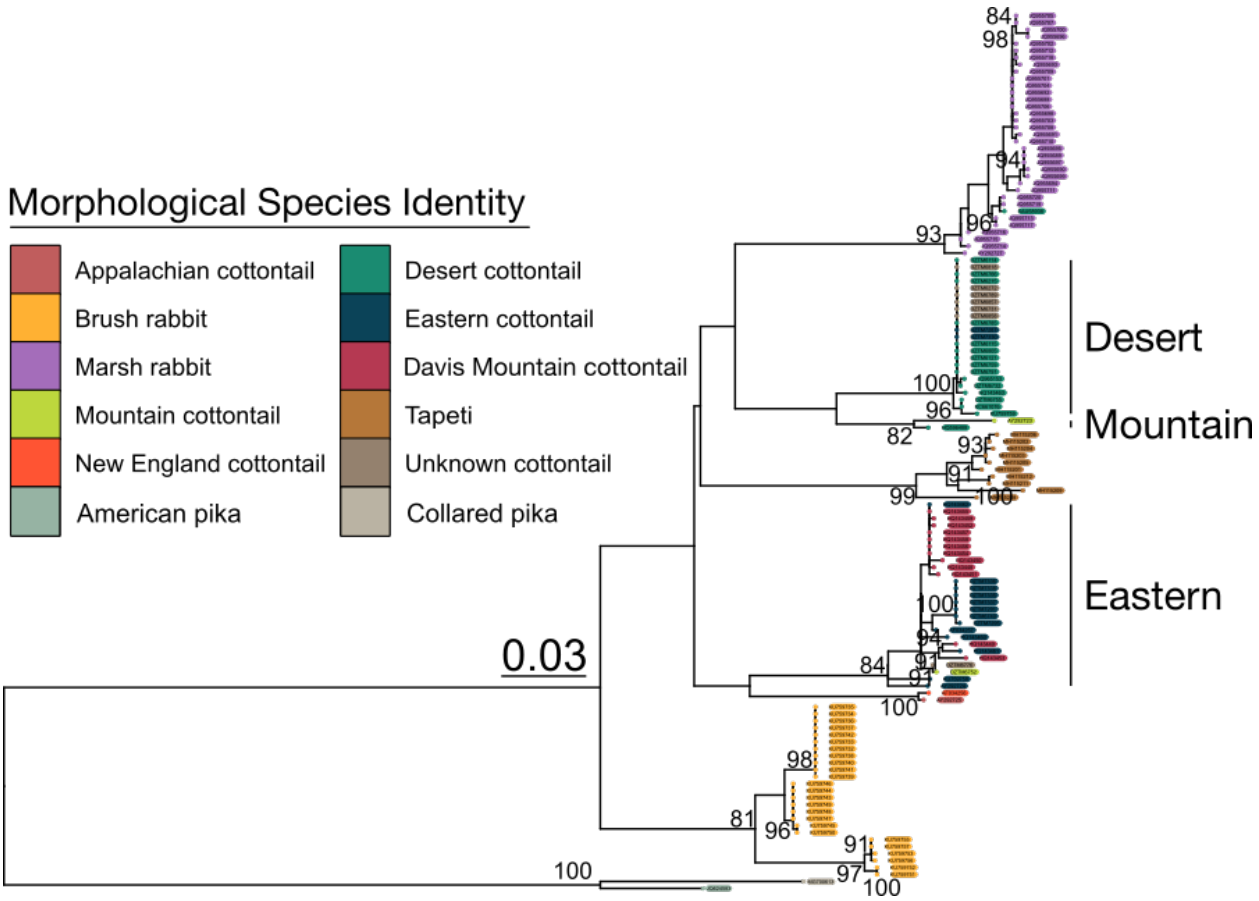

Figure 2. Phylogenetic tree showing grouping of each nine species into clades indicated by color and rooted with pika sequences. Three focal clades, desert, mountain, and eastern cottontails are labelled on the right, with incomplete sorting between the eastern and robust cottontail clades. Scale indicates the number of substitutions per site. Nodes with bootstrap support greater than 80 are labelled.

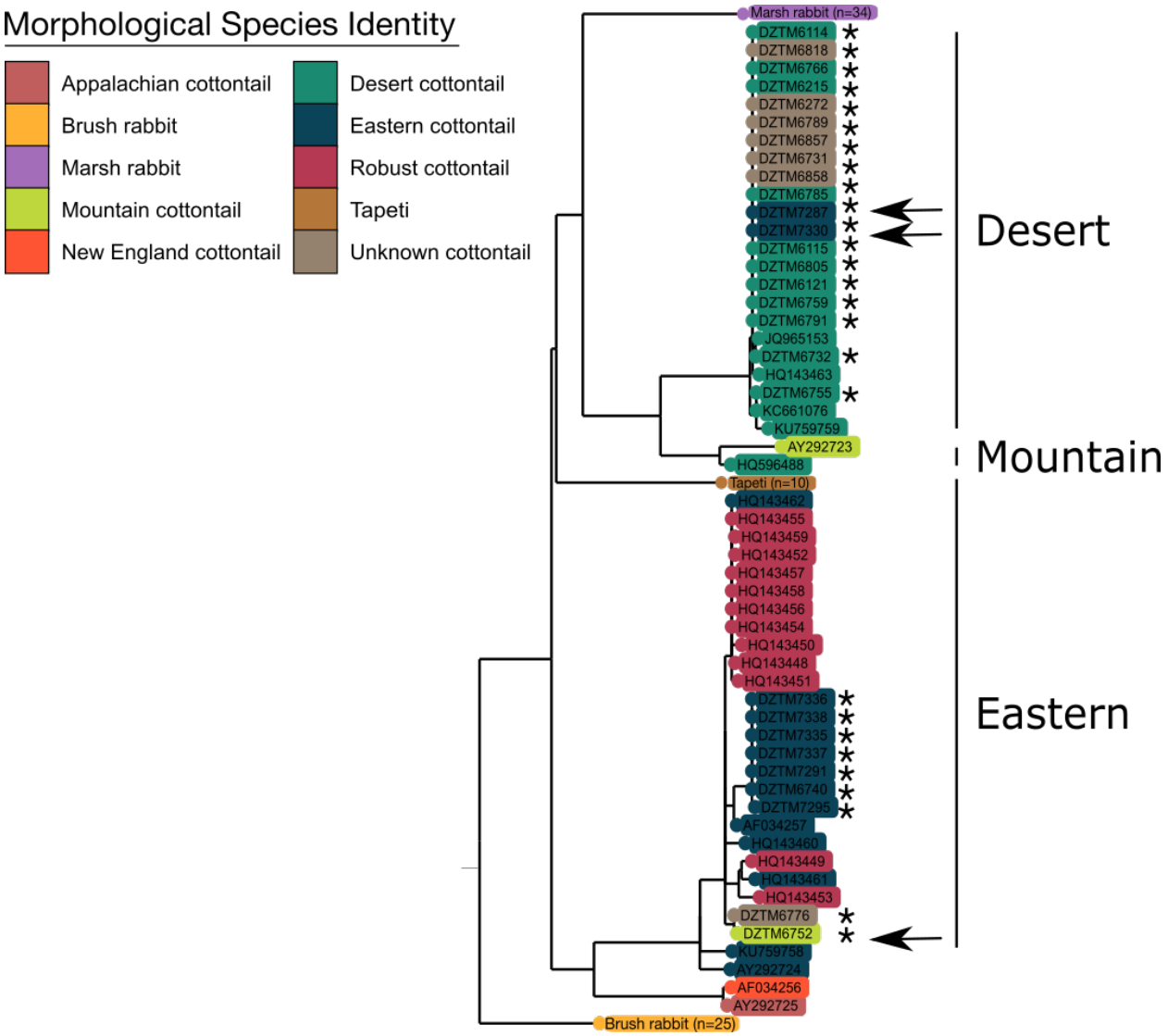

Figure 3. Phylogenetic tree focused on desert, mountain, and eastern cottontail clades. Three focal clades, desert, mountain, and eastern cottontails are labelled on the right and indicate genetic identity. Asterisks (*) indicate which individuals I sequenced and are colored by morphological identity. Arrows indicate where an individual's morphological identity did not align with its genetic identity. Three clades have been collapsed, represented by a single bar, and include the brush rabbit (n = 25), the tapeti (n =10), and the marsh rabbit (n = 34).

Discussion

This study lays the groundwork for understanding species delineation and hybridization in North American cottontails. By focusing on three species that have experienced recent range expansions, likely due to habitat alterations, we can use the cottontail system to understand the impacts of anthropogenic change on species interactions. I found that Colorado cottontails formed two clearly distinct lineages (Figures 2 and 3) and I identified individual cottontails who may be hybrids.

Previously published mtDNA sequences of eastern, desert, and mountain cottontails form three distinct clades relative to mtDNA sequences of other *Sylvilagus* species (Figure 2). However, the Colorado cottontails I sequenced only fell within the desert and eastern clades. The single mountain cottontail individual I sequenced was genetically more similar to eastern cottontails than either desert or mountain cottontails. As shown previously, desert cottontails and mountain cottontails are more closely related to one another than to eastern cottontails (Matthee et al. 2004, Ge et al. 2013). Eastern cottontails are the most divergent lineage of our three focal species, having diverged before desert and mountain cottontails. Our sequencing, along with the reference sequences used to build the phylogenetic tree, support previous phylogenies of the *Sylvilagus* genus (Matthee et al. 2004, Ge et al. 2013). Additionally, eastern cottontails formed a single clade with another species of cottontail, the Davis Mountain (robust) cottontail in agreement with previous studies of the two lineages (Nalls et al. 2012). These lineages are not distinguishable with *cytb*, suggesting the two species may not have fully diverged. However, other, more rapidly evolving *mtDNA* regions such as *12S rRNA* or *d-loop* as well as nuclear loci may be able to differentiate the two.

Overall, morphology was a good indicator of species identity. In most cases, each individual's morphological species identity matched the species mitochondrial genotype. Morphology is often used to define species, as with the Davis Mountain (robust) cottontail (Nalls et al. 2012) and can be a useful way to describe species when genetically distinct lineages are also morphologically distinct. However, it is common for mammal species to be cryptic, which can confound species identification *(e.g.* house mice, Ďureje et al. 2012; chipmunks, Good et al. 2008; and mouse lemurs, Poelstra et al. 2020). In my study, there were seven individuals that were not morphologically identified, and I was able to successfully use mtDNA sequencing to identify the species of these individuals. In our Colorado cottontails, most unknown individuals

grouped with desert cottontails. It is unclear whether this is because desert cottontails are more difficult to identify morphologically or whether they are more morphologically variable than other cottontails. In cases where morphological variation does not clearly delineate species, we need additional lines of evidence to determine species identification more accurately and not rely solely on morphological modes of identification.

While most individuals in this study clustered within the species clade indicated by their morphology, there was a mismatch between the mitochondrial species identification and the morphological species identification for three individuals. It is possible that the three individuals were morphologically misidentified, however, the mismatched mountain cottontail individual was located in Breckenridge, at very high elevation, indicating it is very likely a mountain cottontail. Therefore, it is more likely that the mismatch between morphological identification and mtDNA for these three individuals is due to recent or ongoing hybridization, which is common in mammals (Shurtliff, 2013). With recent or ongoing hybridization, we would expect some mountain individuals to have eastern cottontail nuclear DNA and some desert individuals to have eastern cottontail nuclear DNA. Alternatively, mtDNA capture may be occurring, where mitochondrial markers from other species are introduced to a population through ancient and limited hybridization, which was sufficient for hybridization to persist in the mtDNA but not in nuclear markers. This ancient form of hybridization is also common in mammals, such as chipmunks (Good et al. 2008) and hares (Marques et al. 2017). If mtDNA capture is occurring, we would expect to see geographically widespread evidence of hybridization in the mitochondrial genome of cottontails but limited evidence of introgression in the nuclear genomes of these individuals.

To differentiate between rare hybridization in the past and recent, ongoing hybridization among desert, eastern, and mountain cottontails will require additional work. Sequencing of the *cytb* mitochondrial gene is the first step that must be followed by additional mitochondrial sequencing and nuclear sequencing, and an increased sample size. Additional mitochondrial sequencing should focus on regions that are more variable than *cytb* which will allow us to differentiate between more recently diverged cottontail populations. More nuclear sequencing will provide markers across the entire genome and stronger evidence for introgression. If hybridization is recent and introgression is widespread, we would expect to find individuals with nuclear alleles from each of two different parental species. Lastly, increasing the number of

cottontail individuals will expand the sample size and provide insights into how geographically widespread hybridization is among these lineages. Increasing the sequencing of mountain cottontail individuals is especially important as, so far, my data suggest that no Colorado cottontails have mountain mtDNA. If, upon further sequencing the pattern is maintained, this could indicate widespread hybridization or that all high-elevation cottontails in Colorado are actually eastern cottontails. If no Colorado cottontails are found to have mountain mtDNA, a dramatic redefinition of our estimated species ranges for these two species may be necessary.

The results of this study raise questions about further research that should be conducted. If hybridization is occurring among these three Colorado cottontail species, will it be necessary to redefine species boundaries? Are eastern, desert, and mountain cottontails as divergent as we believe, or are they more closely related than previously suggested? If not, what mating barriers are preventing hybridization from occurring? Alternatively, if introgression of eastern mtDNA into mountain cottontails is found, but no introgression of mountain mtDNA into eastern cottontails, this would indicate only female mountain cottontails are interested in mating with eastern cottontails. The directionality of introgression can give us insight into species barriers. Humans may be interfering with species barriers by bringing habitats together as they continue to develop the west. Evidence of humans breaking species barriers has been found in other mammals such as wolves, coyotes, and chipmunks, through hunting, forest clearing, forest fire, and urbanization (Grabenstein and Taylor, 2018). Further research conducted on the age of the collected samples, whether it is a newer or older sample, can shed light on human impact. If an ancient hybridization event has occurred, we would expect to find older hybrid samples, potentially before we expect eastern cottontails to have arrived. If more recent or ongoing hybridization is occurring, we would expect hybrid samples to be collected more recently from newly urbanized environments. Additionally, expanding the range of sample collection outside of the Rocky Mountain Front Range will help our understanding of human impacts.

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Literature Cited

Barton NH, Hewitt GM. 1989. Adaptation, speciation and hybrid zones. Nature. 341(6242):497–503. doi:10.1038/341497a0.

Bowles J, Blair D, Mcmanus D. 1992. Genetic variants within the genus Echinococcus identified by mitochondrial DNA sequencing. Molecular and Biochemical Parasitology. 54(2):165–173. doi:10.1016/0166-6851(92)90109-W.

Carneiro M, Baird SJE, Afonso S, Ramirez E, Tarroso P, Teotónio H, Villafuerte R, Nachman MW, Ferrand N. 2013. Steep clines within a highly permeable genome across a hybrid zone between two subspecies of the European rabbit. Mol Ecol. 22(9):2511–2525. doi:10.1111/mec.12272.

Davis CM, Roth VL. 2008. The evolution of sexual size dimorphism in cottontail rabbits (Sylvilagus, Leporidae): EVOLUTION OF SSD IN COTTONTAILS. Biological Journal of the Linnean Society. 95(1):141–156. doi:10.1111/j.1095-8312.2008.01035.x.

Ďureje Ľ, Macholán M, Baird SJE, Piálek J. 2012. The mouse hybrid zone in Central Europe: from morphology to molecules. Folia Zoologica. 61(3–4):308–318. doi:10.25225/fozo.v61.i3.a13.2012.

Ge D, Wen Z, Xia L, Zhang Z, Erbajeva M, Huang C, Yang Q. 2013. Evolutionary History of Lagomorphs in Response to Global Environmental Change. Evans AR, editor. PLoS ONE. 8(4):e59668. doi:10.1371/journal.pone.0059668.

Gompert Z, Mandeville EG, Buerkle CA. 2017. Analysis of Population Genomic Data from Hybrid Zones. Annu Rev Ecol Evol Syst. 48(1):207–229. doi:10.1146/annurev-ecolsys-110316-022652.

Good JM, Hird S, Reid N, Demboski JR, Steppan SJ, Martin-Nims TR, Sullivan J. 2008. Ancient hybridization and mitochondrial capture between two species of chipmunks. Mol Ecol. 17(5):1313–1327. doi:10.1111/j.1365-294X.2007.03640.x.

Grabenstein KC, Taylor SA. 2018. Breaking Barriers: Causes, Consequences, and Experimental Utility of Human-Mediated Hybridization. Trends in Ecology & Evolution. 33(3):198–212. doi:10.1016/j.tree.2017.12.008.

Halanych KM, Robinson TJ. 1997. Phylogenetic Relationships of Cottontails (Sylvilagus,Lagomorpha): Congruence of 12S rDNA and Cytogenetic Data. Molecular Phylogenetics and Evolution. 7(3):294–302. doi:10.1006/mpev.1996.0403.

Harrison RG, Larson EL. 2014. Hybridization, Introgression, and the Nature of Species Boundaries. Journal of Heredity. 105(S1):795–809. doi:10.1093/jhered/esu033.

Hasselman DJ, Argo EE, McBride MC, Bentzen P, Schultz TF, Perez-Umphrey AA, Palkovacs EP. 2014. Human disturbance causes the formation of a hybrid swarm between two naturally sympatric fish species. Mol Ecol. 23(5):1137–1152. doi:10.1111/mec.12674.

Irwin DM, Kocher TD, Wilson AC. 1991. Evolution of the cytochromeb gene of mammals. J Mol Evol. 32(2):128–144. doi:10.1007/BF02515385.

IUCN. 2018. Sylvilagus floridanus: Nielsen, C. & Lanier, H.C.: The IUCN Red List of Threatened Species 2019: e.T41299A45191626. doi:10.2305/IUCN.UK.2019-1.RLTS.T41299A45191626.en. [accessed 2021 May 2]. https://www.iucnredlist.org/species/41299/45191626.

Litvaitis MK, Lee W-J, Litvaitis JA, Kocher TD. 1997. Variation in the mitochondrial DNA of the *Sylvilagus* complex occupying the northeastern United States. Can J Zool. 75(4):595–605. doi:10.1139/z97-074.

Marques JP, Farelo L, Vilela J, Vanderpool D, Alves PC, Good JM, Boursot P, Melo-Ferreira J. 2017. Range expansion underlies historical introgressive hybridization in the Iberian hare. Sci Rep. 7(1):40788. doi:10.1038/srep40788.

Matthee CA, Van Vuuren BJ, Bell D, Robinson TJ. 2004. A Molecular Supermatrix of the Rabbits and Hares (Leporidae) Allows for the Identification of Five Intercontinental Exchanges During the Miocene. Sullivan J, editor. Systematic Biology. 53(3):433–447. doi:10.1080/10635150490445715.

Meyer A, Kocher TD, Basasibwaki P, Wilson AC. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. Nature. 347(6293):550–553. doi:10.1038/347550a0.

Nalls AV, Ammerman LK, Dowler RC. 2012. Genetic and Morphologic Variation in the Davis Mountains Cottontail (Sylvilagus robustus). The Southwestern Naturalist. 57(1):1–7. doi:10.1894/0038-4909-57.1.1.

Nucleotide sequence variation in the mitochondrial 12S rRNA gene and the phylogeny of African mole-rats (Rodentia: Bathyergidae). 1992 Jan. Molecular Biology and Evolution. doi:10.1093/oxfordjournals.molbev.a040706. [accessed 2021 May 3]. https://academic.oup.com/mbe/article/9/1/27/986461/Nucleotide-sequence-variation-in-the-mitochondrial.

Poelstra JW, Salmona J, Tiley GP, Schüßler D, Blanco MB, Andriambeloson JB, Bouchez O, Campbell CR, Etter PD, Hohenlohe PA, et al. 2021. Cryptic Patterns of Speciation in Cryptic Primates: Microendemic Mouse Lemurs and the Multispecies Coalescent. Esselstyn J, editor. Systematic Biology. 70(2):203–218. doi:10.1093/sysbio/syaa053.

Shurtliff QR. 2013. Mammalian hybrid zones: a review: Mammalian hybrid zones. Mammal Review. 43(1):1–21. doi:10.1111/j.1365-2907.2011.00205.x.

Smith AT, Johnston, Charlotte H., Alves, Paulo C., Hackländer, Klaus. 2018. Lagomorphs: pikas, rabbits, and hares of the world. Baltimore: Johns Hopkins University Press.

Taylor SA, Larson EL, Harrison RG. 2015. Hybrid zones: windows on climate change. Trends in Ecology & Evolution. 30(7):398–406. doi:10.1016/j.tree.2015.04.010.

Taylor SA, White TA, Hochachka WM, Ferretti V, Curry RL, Lovette I. 2014. Climate-Mediated Movement of an Avian Hybrid Zone. Current Biology. 24(6):671–676. doi:10.1016/j.cub.2014.01.069.

Verts BJ, Carraway LN. 1980. Natural Hybridization of Sylvilagus bachmani and Introduced S. floridanus in Oregon. The Murrelet. 61(3):95. doi:10.2307/3535190.

Yoder AD, Campbell CR, Blanco MB, dos Reis M, Ganzhorn JU, Goodman SM, Hunnicutt KE, Larsen PA, Kappeler PM, Rasoloarison RM, et al. 2016. Geogenetic patterns in mouse lemurs (genus *Microcebus*) reveal the ghosts of Madagascar's forests past. Proc Natl Acad Sci USA. 113(29):8049–8056. doi:10.1073/pnas.1601081113.