# Genetic population structure and relatedness of Colorado mule deer (*Odocoileus hemionus*) and incidence of chronic wasting disease

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ABSTRACT: Understanding genetic variation among individuals is important to understanding disease transmission. In particular, genotypes are believed to influence susceptibility of mule deer (*Odocoileus hemionus*) to chronic wasting disease. We examined the genetic structure, PrP genotypes and genetic relationships of mule deer in north-central Colorado in relation to occurrence of chronic wasting disease (CWD). Genotypes from 8 microsatellite loci in 250 mule deer captured between January 2003 and April 2005 in Estes Valley, Colorado, Middle Park, Colorado and on the west side of the continental divide in Rocky Mountain National Park, Colorado were analyzed based on sampling location, sex, and CWD status. The fixation index, $F_{ST}$, and differences in allele frequency distributions provided evidence of significant difference between sampled groups ($F_{ST}$=0.0092, S.E.=0.0003, *P*=0.0499; allele frequency *P*=0.015). These results were primarily attributed to differences observed between the Middle Park and Estes Valley sampled groups ($F_{ST}$=0.0118, S.E.=0.0052, *P*=0.015; allele frequency *P*=0.00134). Analyses conducted on each sex separately revealed that only females had significant multilocus differences between Middle Park and Estes Valley ($F_{ST}$=0.0174, S.E.=0.0085, *P*=0.043; allele frequency *P*=0.0011). When deer were analyzed based on CWD status we found little evidence of genetic structure by CWD status. We also found that there were fewer CWD-positive deer than expected in the heterozygous serine/phenylalanine genotype at codon 225 of the PrP gene and more CWD-positive deer than expected in the homozygous serine genotype, however, these differences were only significant in males (*P*=0.020). There was no evidence that CWD-positive deer as a group or CWD-positive males were more closely related to each other than would be expected based on the reference population (all deer, *P*=0.20; males *P*=0.98), however,

we found that CWD-positive females were more closely related to each other than expected ($P$=0.027). Our results indicate that 1) there is genetic differentiation between deer wintering in Estes Valley and Middle Park but these differences appear attributable to females indicating that mixing of populations is likely dominated by males, 2) genotypes and genetic structure based on the eight microsatellite loci in our study did not reveal any genotypes that were specifically associated with CWD, 3) there were more CWD-positive males observed with the homozygous serine genotype and less observed with the heterozygous serine/phenylalanine genotype than expected, and 4) CWD-positive female deer are more closely related than expected supporting the hypothesis that once a member of a female matrilineal group becomes infected with CWD it is passed to other closely related females within the group.

*Key words: Odocoileus hemionus*, mule deer, chronic wasting disease, relatedness, population structure, kinship, microsatellites

## INTRODUCTION

Individual genotypes, genetic structure and genetic relationship have been used to study a variety of questions that may impact disease transmission. These have included mule deer genotypes in relation to susceptibility and incubation of chronic wasting diseaese (Jewell et al., 2005) and social group composition in large grazing mammals (Archie et al., 2006). White-tailed deer (*Odocoileus virginianus*) social structure has been well studied and mule deer (*Odocoileus hemionus*) are suspected to have the same dynamics. Deer social structure includes females forming philopatric matrilineal groups and males either dispersing individually or in groups with unrelated males (Aycrigg and Porter, 1997). Mule deer exhibit male-biased natal and breeding dispersal (Greenwood, 1980) with little evidence of female dispersal in Colorado (Connor and Miller, 2004). These patterns may favor kin selection (Hamilton, 1964) when groups of related females cooperate in predator defense and rearing of young, as well as reduce inbreeding (Greenwood, 1980) when related males avoid breeding with close relatives through dispersal. These same dynamics may facilitate the spread of chronic wasting disease (CWD).

Chronic wasting disease is spread through oral exposure (Sigurdson et al., 1999) that can occur from maternal (dam to offspring), horizontal (animal to animal), or environmental contacts (Miller and Williams, 2003; Miller et al., 2004). There is evidence that CWD infection and length of incubation may also be influenced by deer genotype at codon 225 of the PrP gene (Jewell et al., 2005). Once CWD is introduced into a female matrilineal group horizontal contacts could increase the spread of the disease to other related group

members. Dispersal by CWD-positive males may introduce CWD into previously unexposed groups of deer.

We sought to examine genetic structure and genetic relationships of mule deer in north-central Colorado. Genetic structure, relationship, and individual PrP genotypes were also compared to patterns of CWD infection. Deer were sampled on both the east and west sides of the continental divide to determine genetic exchange across a broader landscape and to examine the role the continental divide may play in limiting gene transfer. Our primary questions were: 1) Are deer in Estes Valley, Middle Park and West RMNP genetically distinct? Is there evidence of dispersal or gene transfer across the continental divide? 2) Are CWD-positive deer genetically distinct from CWD-negative deer based on multilocus microsatellite genotypes? 3) Are PrP genotypes at codon 225 independent of CWD infection? 4) Are CWD-positive deer more closely related to one another than to the rest of the population?

## MATERIALS AND METHODS

### Study area

Our primary study area was located at 40°22'30"N, 105°30'00"E, encompassing 35 $km^2$ in the Estes Valley including Larimer County, Colorado, between 7,000 and 10,000 feet in elevation. The western portion of this study area included Rocky Mountain National Park; the central and eastern portions of the study area were primarily private lands. Mule deer habitat includes the montane zone where open ponderosa pine (*Pinus ponderosa*) stands predominate. Our extended study area included 33 $km^2$ on the west

side of the continental divide in Rocky Mountain National Park, located at 40°23'30"N, 105°52'00"E, and ranging from 8,500 to 11,000 feet in elevation along the Kawuneeche Valley. This habitat varies from Engelmann spruce (*Picea engalmanni*) and subalpine fir (*Abies lasiocarpa*) forests to riparian areas dominated by willow (*Salix spp.*). The third area where deer were sampled was located at 40°06'00" N, 106°20'00"E, and included a 270 $km^2$ section of winter range in Middle Park, Colorado, below 9,000 feet in elevation and dominated by big sagebrush (*Artemesia tridentata*).

In our study area female groups of mule deer on summer range (June 15-September 30) typically consist of one doe with one to two fawns but larger groups of up to seven deer have been observed (Harrington, 1978). On winter range (December 1 – February 28) female group size increases averaging of 6.7 members (Capp, 1967). Groups of males on summer range are more variable with group size from one to eight (Harrington, 1978). On winter range male groups average 1.8 individuals (Stevens, 1980). The relationship of group members to one another has not been studied in our area. Dispersal rates in north-central Colorado are low (2.0%) with no dispersal documented in our sampling areas (Connor and Miller, 2004).

**Mule deer capture and sample collection**

Genetic samples were submitted for genotyping from 290 mule deer that were captured between January 2003 and April 2005 (Figure 1). Deer were primarily captured in the contiguous areas of Rocky Mountain National Park (RMNP) east of the continental divide, the town of Estes Park, and the surrounding Estes Valley. These groups will be

referred to collectively as Estes Valley. Deer were also captured in Middle Park and in RMNP on the west side of the continental divide (West RMNP). Deer from the Estes Valley and West RMNP were captured using free-range darting. These deer were anesthetized using a variety of combinations of thiafentanil (ZooPharm, Fort Collins, Colorado, USA), medatomidine (ZooPharm, Fort Collins, Colorado, USA), tiletamine and zolazepam (Telazol®, Fort Dodge Animal Health, Fort Dodge, Iowa, USA), xylazine HCl (MWI, Meridian, Idaho, USA) and ketamine (ZooPharm, Fort Collins, Colorado, USA). Inside of RMNP deer were captured by National Park Service staff (Colorado State University ACUC Protocol Approval Numbers: 02-311A-01 through 02-311A-03). In Estes Park deer were captured by the Colorado Division of Wildlife (Colorado Division of Wildlife ACUC Protocol Approval Number: 05-2003). Deer in Middle Park were captured for routine wildlife management activities using helicopter net gunning under the direction of the Colorado Division of Wildlife. Middle Park deer were physically restrained without use of anesthesia.

While deer were under chemical or physical restraint, blood was drawn from the jugular vein and preserved in whole blood tubes with 15% liquid EDTA (Sherwood Medical, Saint Louis, Missouri, USA). Samples were typically kept at ambient temperatures for one to five hours after collection but in late spring and early fall samples were placed in a cooler with ice to avoid exposure to high temperatures for extended periods of time. Blood samples were either extracted immediately or placed in a -12°C freezer for short-term storage. Both extracted DNA and whole blood were transferred to a -62° C freezer for permanent storage. Hair samples were also collected from deer captured in RMNP.

Hair was collected by pulling a small patch of hair either using a sterile gloved hand or small pliers and immediately inserting the hair into a manila coin envelope follicle-side down. Envelopes were triple-bagged in sealed plastic bags and stored in a refrigerator at 6°C. Tonsil biopsies were performed on mule deer from all areas except Middle Park to determine if they had chronic wasting disease (CWD). Collection of tonsil biopsy samples generally followed the techniques that have been previously described (Wild et al., 2002). Our samples included two to three individual samples of tissue collected dorso-laterally from the tonsil and tonsil crypt using Jackson biopsy forceps (Sontec Instruments, Englewood, Colorado, USA) with a six mm cup. Tissue was preserved in 10% buffered neutral formalin. Samples were submitted to the Colorado State University Veterinary Diagnostic Laboratory or the Wyoming State Veterinary Laboratory for CWD testing.

**DNA extraction and genotyping**

DNA was extracted from the whole blood of mule deer by the Colorado Division of Wildlife (CDOW) or the University of California, Los Angeles, Conservation Genetic Resource Center (UCLA). The Animal Disease Research Unit in the Agricultural Research Service of the U. S. Department of Agriculture (ARS) extracted DNA from the hair samples. The extraction methods varied by laboratory. The CDOW extracted DNA using the Qiagen DNeasy Blood and Tissue Extraction Kit (QIAGEN Inc., Valencia, CA) using the Animal Blood (Spin-Column) Protocol and adjusting the blood volume to 220ul for extraction (100ul of blood, 100ul of PBS and 20ul of proteinase K). The final DNA elution was in 200ul of Buffer AE which is 10 mM Tris-Cl, 0.5mM EDTA, pH 9.0. The

UCLA Conservation Genetic Resource Center extracted DNA from whole blood using Qiagen QIAamp DNA Mini kits (QIAGEN Inc., Valencia, CA) and exactly following the manufacturer's protocols. DNA was extracted by the ARS using commercial kits (Q-Biogene, Carlsbad, CA, USA or QIAGEN Inc., Valencia, CA) and following the manufacturer's instructions with slight revisions.

UCLA conducted microsatellite genotyping from the extracted blood samples. Sixteen known mule deer markers were initially examined for their suitability in genotyping deer from our study area. Microsatellite markers were amplified using PCR with dye labeled primers. Markers were multiplexed in sets of either three or four markers using Qiagen Multiplex Mix PCR reagents (QIAGEN Inc., Valencia, CA). The resulting product was characterized on an ABI3700 capillary sequencer and analyzed using ABI Genemapper 3.7 software. The PCR reactions utilized a hybrid combination of forward primers consisting of the published forward primer with an M13F (-20) sequence (16 bp) added to the 5' end and a fluorescent dye labeled M13F (-20) primer. The reverse primer was unlabeled. ARS genotyped 129 mule deer from the Estes Valley at codons 60, 95, 96, 116, 131, 146, 156, 202, and 225 of the PrP gene. PCR amplification was performed using a primer pair specific for amplification of the prion gene (forward primer 223 5'-aca ccc tct tta ttt tgc ag - 3' and reverse primer 224 5'-aga aga taa tga aaa cag gaa g - 3'). The PCR reaction was as follows: 95$^{o}$C for 5 min, followed by 30 cycles of denaturation (95$^{o}$C, 30 s), annealing (54$^{o}$C, 30 s) and extension (72$^{o}$C, 30 s) followed by an extension cycle (72$^{o}$C, 7 min) under standard buffer conditions with 2.5mM $MgCl_2$ (QIAGEN, Inc., Valencia, CA). PCR products were purified by Exo/SAP to remove unincorporated

dNTPs and primers, then sequenced using forward primer 245 (5'-ggc aac cgc tat cca cct ca- 3') and reverse primer 12 (5'-tgg tgg tga ctg tgt gtt gct tga-3') using Big Dye chemistry (ABI) (Amplicon Express, Pullman, WA USA). An additional 83 study animals from the Estes Valley had been previously genotyped at codon 225 of the PrP gene by the Wyoming State Veterinary Laboratory (Jewell et al., 2005).

**Marker selection**

Five assumptions are important when using microsatellite markers for genetic population structure (Pritchard et al., 2000) or relatedness analysis (Lynch and Ritland, 1999). Microsatellite markers were selected based on adherence to the following assumptions: 1) there is random union of gametes, 2) loci are selectively neutral, 3) there is an absence of null alleles, 4) there are low levels of mutation, and 5) loci are unlinked. The first four assumptions were tested by examining loci for conformance with Hardy-Weinberg expectations. Sixteen candidate loci were tested by UCLA for deviation from Hardy-Weinberg equilibrium (HWE) using the software program GENEPOP 3.4 (Raymond and Rousset, 1995a). Six markers were eliminated because they were heterozygote deficient indicating possible null alleles.

We tested the remaining ten loci in each sampled area (Estes Valley, West RMNP, and Middle Park) for HWE using the exact test of Guo and Thompson (1992) and for concordance with the fifth assumption, unlinked loci. Our initial assumption was that each of these sampling areas represented a separate population and therefore loci for each area should be in HWE and linkage equilibrium. Tests were performed with the software

package GENEPOP 3.4 (Raymond and Rousset, 1995a). Markov chain parameters were set to 1,000 for dememorization, 1,000 batches and 10,000 iterations per batch. Bonferroni corrections were made to significance levels to account for multiple comparisons. The final markers are reported with allelic diversity, observed heterozygosity, expected heterozygosity and probability of significant departure from HWE calculated in GenAlEx 6 and GENEPOP 3.4 (Raymond and Rousset, 1995a; Peakall and Smouse, 2006; Table 1).

**Chronic wasting disease determination**

CWD status was determined using immunohistochemistry on brain, tonsil or retropharyngeal lymph nodes following the methods previously reported (Spraker et al., 2002). Positive immunostaining indicated a deer was CWD-positive and no staining indicated a deer did not have detectable levels of CWD.

**Analysis of genetic population structure**

The straight-line geographic distances between the approximate midpoints of each sampled area were 50 km between Middle Park and West RMNP, 65 km between Middle Park and Estes Valley and 25 km between West RMNP and Estes Valley. The Middle Park and West RMNP sampling areas are west of the continental divide and the Estes Valley sampling area is east of the continental divide. In order to study whether distance and/or topography resulted in population substructure between these sampling areas we analyzed groups of deer using *F*-statistics and differences in allele frequencies.

We examined hierarchical population structure by measuring the fixation index, $F_{ST}$. We calculated $F_{ST}$ for each sex separately and for each sampling group comparison using the method of Weir and Cockerham (1984) in program SPAGeDi (Hardy and Vekemans, 2002). This method accounts for unequal sample sizes and uses a weighted average over alleles at each locus and over all loci (Weir and Cockerham, 1984). We jackknifed over loci to determine the mean and standard errors for multilocus statistics. For each comparison a permutation procedure of individuals among all populations was used and the number of permutations was set to 10,000. This allowed us to test if differences in heterozygosity were significant under the null hypothesis, $H_O$: observed $F_{ST} = F_{ST}$ after 10,000 permutations of individuals among all populations, against the alternative hypothesis, $H_1$: observed $F_{ST} \neq F_{ST}$ after 10,000 permutations of individuals among all populations. If genetic structure is influenced by distance we would expect to see the greatest reduction in heterozygosity and most significant differences between observed and $F_{ST}$ after 10,000 permutations of individuals among all populations between the Estes Valley and Middle Park sampling areas and the least reduction in heterozygosity and non-significant differences in observed and $F_{ST}$ after 10,000 permutations of individuals among all populations between Estes Valley and West RMNP. If topography is responsible for genetic differentiation we would expect to see the greatest reduction in heterozygosity and significant differences between observed and $F_{ST}$ after 10,000 permutations of individuals among all populations between Estes Valley and Middle Park and Estes Valley and West RMNP which are on opposite sides of the continental divide but little reduction in heterozygosity and non-significant differences in observed and $F_{ST}$

after 10,000 permutations of individuals among all populations between West RMNP and Middle Park which are on the same side of the continental divide.

We also looked for population differentiation by testing for differences in allele frequencies between populations. These tests were conducted in the software package GENEPOP 3.4 (Raymond and Rousset, 1995a). Once again tests were run for all individuals, males only and females only between each sampling area (Estes Valley, West RMNP and Middle Park). Markov chain parameters were set to 1,000 for dememorization, 1,000 batches and 10,000 iterations per batch. The null hypothesis tested was, Ho: The allelic distribution is identical across sampling areas. Fisher's exact test provided an unbiased estimate of the *P*-value for each locus and for all loci combined (Raymond and Rousset, 1995b).

The Estes Valley sampling area was the only group with CWD-positive deer. In order to look for genetic differences between CWD-positive and negative deer we examined the genetic structure of the Estes Valley group separately. We looked for $F_{ST}$ and allele frequency differences between CWD-positive and CWD-negative deer using the same methods described above for all populations.

**Analysis of PrP genotype and chronic wasting disease**

Genotypes at codon 225 of the PrP gene were compared with CWD status in deer. Two genotypes were observed and deer could only be CWD-positive or CWD-negative, therefore, we created 2x2 tables of these two variables using the PROC FREQ command

in SAS 9.1 (SAS Institute Inc., 2002-2003; Scholtzhauer and Littell, 1997). We then tested the null hypothesis, $H_O$: CWD infection is independent of codon 225 PrP genotype, against the alternative hypothesis, $H_1$: CWD infection is not independent of codon 225 PrP genotype, using Fisher's exact test.

**Analysis of relatedness and chronic wasting disease**

Relatedness of deer was only analyzed for the Estes Valley group because it was the only group with CWD-positive sampled animals. We wanted to determine whether CWD-positive deer were more closely related to each other than would be expected based on the reference population. Relatedness comparisons were conducted for all CWD-positive and CWD-negative deer as well as separately for CWD-positive and CWD-negative females and males. Pairwise relationship co-efficients (r) were calculated using a method of moments estimator with an underlying regression model (Lynch and Ritland, 1999) in program GenAlEx 6 (Peakall and Smouse, 2006). Relationship co-efficients were calculated by using the both ways and mean option, which uses each individual of a pair as the reference individual and then takes the mean of the two co-efficients. The average population relationship co-efficient for CWD-positive and CWD-negative groups of deer were calculated using the Pops Means option in GenAlEx 6 (Peakall and Smouse, 2006) and setting parameters to 9,999 bootstraps to establish the 95% confidence interval around the mean population co-efficient and 10,000 permutations to test for significance. A relationship co-efficient near zero indicates a neutral relationship. As co-efficients increase toward one this indicates an increasing relationship. An r-value of 1.0 indicates monozygotic twins, 0.5 indicates a first-order relationship such as parent-offspring or full

siblings and 0.25 indicates a second order relationship such as half siblings, grandparent-grandchild or aunt-niece. The null hypothesis, $H_O$: There is no difference between the mean relatedness of CWD-positive deer and the reference population, was tested against the alternative hypothesis, $H_1$: The mean relatedness of CWD-positive deer is greater than the mean relatedness of the reference population.

## RESULTS

### DNA extraction and genotyping

Out of the 16 initial microsatellite markers tested, six were not in conformance with Hardy-Weinberg equilibrium for multiple sampling locations and were eliminated. Deer were genotyped at the remaining ten candidate loci. All ten markers did not amplify in all deer. We only considered results for deer with genotypes that amplified at seven or more loci. Of the original 290 samples, 250 deer met this requirement. The 250 deer genotyped included 220 from Estes Valley, five from West RMNP and 25 from Middle Park. These 10 loci were then further tested to see if they were appropriate markers for the population genetic structure and relatedness analyses.

PrP genotypes were identified for 129 of the 131 deer samples submitted from Estes Valley. The genotypes at codon 225 of the PrP gene of 83 deer were known from a previous study (Jewell et al., 2005). Therefore, 212 of the 220 study animals from Estes Valley had a known genotype at this marker. There are three possible genotypes at codon 225. Deer can be homozygous for serine (S/S), homozygous for phenylalanine (F/F) or

heterozygous (S/F). Of our 212 deer, 37 were heterozygous S/F and the remaining 175 were homozygous S/S. None of our deer had the F/F genotype.

**Marker selection**

Tests for HWE revealed that at locus C10 the null hypothesis, $H_O$=There is random union of gametes, could not be rejected for West RMNP and Middle Park but was rejected for Estes Valley ($P$<0.0001). Therefore, locus C10 was eliminated as a viable marker. The remaining nine loci were in HWE for all sampled areas. After C10 was eliminated, the nine remaining candidate loci were tested for linkage equilibrium. The null hypothesis, $H_O$=Genotypes at one locus are independent from genotypes at the paired locus, was rejected for loci C217 and T217 in the Middle Park and Estes Valley sample areas ($P$<0.001 for both groups). T217 had failed to amplify in more deer than C217 and locus T217 was closer to the rejection zone for HWE than C217. For these reasons T217 was eliminated as a candidate marker and C217 was retained. The remaining eight markers were in linkage equilibrium for Middle Park, West RMNP and Estes Valley. The final marker set is listed in Table 1.

**Chronic wasting disease determination**

Of the 250 deer with microsatellite genotypes, 32 were identified as CWD-positive. The 32 CWD-positive deer included 18 males and 14 females. All CWD-positive deer were in the Estes Valley sampled group.

**Analysis of genetic population structure**

We looked at $F_{ST}$ between our predefined groups of Estes Valley, West RMNP, and Middle Park (Table 2). $F_{ST}$ values across all loci and sampling areas indicated little reduction in heterozygosity ($F_{ST}$=0.0092, S.E.=0.003) between groups but these differences were significant (*P*=0.0499; Table 2). When only males or females were examined little reduction in heterozygosity was observed and differences were not significant. Pairwise comparisons between the sampling areas of Middle Park and West RMNP as well as Estes Valley and West RMNP revealed little reduction in heterozygosity and non-significant differences between observed and $F_{ST}$ after 10,000 permutations of individuals among all populations for all deer, males only and females only. However, significant differences were observed between Estes Valley and Middle Park for all deer combined across all loci ($F_{ST}$=0.0118, S.E.=0.0052, *P*=0.015). There were no significant differences observed between Middle Park and Estes Valley males across all loci ($F_{ST}$=0.0046, S.E.=0.0083, *P*=0.51). Females in these two areas were significantly different across all loci ($F_{ST}$=0.0174, S.E.=0.0085, *P*=0.043).

Allele frequency distributions provided additional evidence that Estes Valley, Middle Park and West RMNP were significantly different across all loci (*P*=0.015). As with the $F_{ST}$ results these differences were primarily attributable to Estes Valley and Middle Park which had significant differences at four of eight loci as well as across all eight loci (*P*=0.00134). Differences were not significant for comparisons between Estes Valley and West RMNP or between Middle Park and West RMNP at any loci for either sex which also corresponded with the $F_{ST}$ results. When males from Middle Park and Estes Valley

were examined alone, allele frequency distributions were not significant across all loci (*P*=0.078). Comparisons of females only in Estes Valley and Middle Park revealed that multilocus allele frequency distributions were significantly different (*P*=0.0011).

When comparing groups by CWD status we found little evidence of genetic structure between CWD-positive and CWD-negative deer. $F_{ST}$ values indicated there were small but non-significant reductions in multilocus heterozygosity between hierarchical levels for males ($F_{ST}$ =-0.0029, S.E.=0.0045, *P*=0.66), females ($F_{ST}$=0.0044, S.E.=0.0109, *P*=0.47) and both sexes combined ($F_{ST}$=-0.0005, S.E.=0.0018, *P*=0.98). Allele frequencies were also not significantly different across all loci for any group (males, *P*=0.38; females, *P*=0.71; both sexes combined, *P*=0.34).

**Analysis of PrP genotypes and chronic wasting disease**

Of the 250 deer in our study 212 had a CWD test result and had been genotyped at codon 225 of the PrP gene (Table 3). When both sexes were combined observed genotypes for CWD-positive and CWD-negative deer were not significantly different than expected (*P*=0.12). When only females were tested the observed and expected frequencies were nearly equal and differences were not significant (*P*=0.69). However, in males CWD status and codon 225 genotype were not independent of one another and these differences were significant (*P*=0.020). Specifically, there were more CWD-positive males observed with the S/S genotype than expected and fewer CWD-positive males observed with the S/F genotype than expected.

### Analysis of relatedness and chronic wasting disease

Of the 220 deer with microsatellite genotypes in Estes Valley, 216 had a known CWD status. Pairwise relatedness co-efficients and group means were compared for both sexes combined, males only, and females only. There were 32 CWD-positive deer (18 males, 14 females) and 184 CWD-negative deer (87 males, 97 females). The null hypothesis, $H_O$: There is no difference between the mean relatedness of CWD-positive deer and the reference population, could not be rejected for both sexes combined ($P$=0.20) or for males ($P$=0.98, Table 4). However, the null hypothesis was rejected for CWD-positive females, with CWD-positive females related more closely than expected ($P$=0.027, Table 4).

## DISCUSSION

From our analyses we had conflicting results in determining whether Estes Valley, Middle Park and West RMNP were genetically distinct from one another. Results differed by sex. Some level of structure was evident in *F*-statistics and allele frequency distributions. We found no evidence to support differences between Middle Park and West RMNP or between the Estes Valley and West RMNP for males, females or all deer combined. The overall lack of evidence of genetic differentiation in males between Estes Valley and Middle Park was surprising. These areas lie 65 km apart on opposite sides of the continental divide and serve as separate winter ranges. Deer typically begin arriving on their winter range in September and October (Connor and Miller, 2004) and breeding normally takes place on these separate ranges which would result in geographically and biologically limiting the gene flow between Estes Valley and Middle Park.

One explanation could be an overall lack of genetic diversity for deer in our study which would make differentiating between populations difficult. Our two primary sampled areas of Estes Valley and Middle Park had lower multilocus observed heterozygosity than expected. The reduction in multilocus heterozygosity of individuals as compared to the overall population ($F_{IT}$) accounted for only 4.4% of the overall genetic variation but this difference was significant ($F_{IT}$=0.0446, S.E.=0.0148, *P*=0.0019). Previous protein starch electrophoresis studies of mule deer in Colorado failed to find significant genetic differences between groups of deer even when studying populations that had much greater geographic separation than our study (Smith et al., 1990; Scribner et al., 1991). Other genetic studies that used microsatellite markers in mule deer and subspecies of mule deer are limited but indicate that our observed heterozygosity of 0.6413 and number of alleles per locus of 6.625 are within the range of reported values. North-central Colorado mule deer had higher levels of heterozygosity and alleles per locus than reported for deer from Utah, Arizona, and Texas combined (heterozygosity=0.480, alleles per locus=4.92) but lower allelic diversity than California (alleles per locus=10.25; Engel et al., 1996; Jones et al., 2000). Lack of genetic diversity could have resulted from the rapid reduction of the mule deer population in the Estes Valley between 1860 and 1900. Mule deer were greatly reduced as a result of competition with domestic livestock, human settlement including roads and fences encroaching on wildlife range and market hunting to supply mining camps (Packard, 1947). Market hunting of mule deer also occurred in the Middle Park Basin and mule deer were at their lowest levels throughout the west during the first three decades of the twentieth century (Gill et al., 2001).

Although deer declined they were not completely extirpated (Stevens, 1980). Therefore populations may have gone through a population bottleneck with the corresponding loss of alleles and lowering of genetic diversity. Although this could explain a relative lack of diversity in each population it would be unlikely for two separate areas to go through a bottleneck, lose similar alleles and therefore appear as one population. However, if one population had several migrants that re-established the second population then it is possible that both populations would be genetically similar. This explanation cannot account for the opposing results we found between males and females. A bottleneck should affect both sexes equally rather than reducing genetic differences in males but allowing for genetic differences to remain in females. Differences in hunting pressure by sex could explain this discrepancy but only if females were more aggressively hunted. Hunting pressure, however, is traditionally greater on males than females until population reduction becomes the key objective (Gill et al., 2001), therefore, differential hunting pressure cannot adequately explain our results.

A more plausible explanation is male-biased dispersal of deer between these two areas. Previous studies in Estes Valley and Middle Park have not found evidence of dispersal (Connor and Miller, 2004) but no study has had the specific objective of quantifying dispersal. Yearling male deer who are the most likely to disperse have been underrepresented in previous studies in north-central Colorado (Greenwood, 1980; Cronin et al., 1991; Connor and Miller, 2004). Our analyses do provide some evidence of male dispersal. Although males and females both showed little genetic differentiation between Estes Valley and Middle Park, the differences observed in females were

significant while those in males were not. Our most sensitive indicator of population differentiation, allele frequency distributions, revealed that unlike females, males in the Estes Valley and Middle Park groups were not significantly different from one another (males, $P$=0.078; females, $P$=0.0011). These discrepancies can be explained by male biased dispersal which would result in mixing of males in these populations that may have gone unrecognized in previous observational studies.

If dispersal of males is indeed occurring it highlights the concern of CWD transmission from Estes Valley to Middle Park deer. The prevalence of CWD on the east side of the continental divide including Estes Valley is 6.9% (C.I. 4.94-8.90; n=636), almost 17 times higher than on the west side of the continental divide in the Middle Park area where prevalence is 0.41% (C.I. 0.00-0.088, n=730; Miller, 2003). Chronic wasting disease increases with age and the highest disease prevalence is observed in five to six year old males (Miller and Connor, 2005). Males typically disperse at age one (Greenwood, 1980). During a recent five-year CWD live "test and cull" study in the Estes Valley, 2.4% (one of 41) of the yearling males tested in RMNP were CWD-positive. This does not eliminate concern about disease transmission from Estes Valley to Middle Park but it does provide evidence that age of males at dispersal does not typically coincide with the age when males are most likely infected with CWD. If males are dispersing from Middle Park to Estes Valley they increase their exposure to CWD by dispersing to areas with higher infection rates. Males likely have an increased risk of exposure and transmission of CWD because of their breeding behavior which involves close contact with many females.

Distance rather than topography appears to explain the results from pairwise comparisons for each of our three groups. Estes Valley and West RMNP are on opposite sides of the continental divide but are in closest geographic proximity out of our three sampled areas. We could find no evidence of genetic differentiation between these two groups. Conversely, Estes Park and Middle Park are the most distantly separated groups in our study. Both $F_{ST}$ and allele frequency differences were significant across all loci and individuals. These results support that distance rather than the continental divide is the barrier to gene flow. However, the West RMNP sample size (n=5) is extremely small and four of the five individuals sampled were female. Our sampling size was likely too small to give an accurate picture of the West RMNP genetic composition particularly in terms of allelic diversity. Additionally, all five deer were captured in August prior to moving to their winter range. Approximately 15-20% of Estes Valley wintering deer move to summer range that is close to or across the continental divide (Conner, 2004). Two of the West RMNP deer were subsequently documented in the Estes Valley indicating that these individuals may actually be a part of the Estes Valley group. For these reasons our results should be interpreted with caution and the role of the continental divide in limiting gene flow should be further explored.

Chronic wasting disease positive deer were not genetically distinct from CWD-negative deer based on multilocus microsatellite genotypes. Our PrP genotype results were able to support previous research that at codon 225 of the PrP gene there were more CWD-positive deer observed with the S/S genotype and fewer observed with the S/F genotype

than expected but for male deer only (Jewell et al., 2005). Results from only females and from all deer combined were not able to support the previous research. Our sample size if 212 was much smaller than the original study (Jewell et al., 2005; n=1482) which may have accounted for this difference. It is also possible that on a smaller landscape scale where the majority of deer were sampled, PrP genotype at codon 225 may not play as large role in CWD susceptibility and incubation as social group interactions and environmental sources of CWD.

There was evidence that CWD-positive females tend to be more closely related to each other than expected (r=0.018, *P*=0.027). Similar evidence was not found in males and is probably attributable to presumed matrilineal structure. If females do stay in closely related groups it is reasonable to expect that exposure to CWD could happen concurrently with many group members through foraging in areas where CWD is present in the environment, mating with the same infected male or similar exposure to an infective group member. Once one member of the group becomes infectious they would have frequent contact and interaction with other members of their kin group which would likely result in other group members becoming infected. Because males are not known to form kin groups and breeding with related females is typically avoided (Greenwood, 1980) it is not surprising that we do not see increased relatedness among CWD-positive males.

Our research shows that male deer in north-central Colorado form one panmictic population while females in the separate wintering groups of Middle Park and Estes Valley show genetic differentiation. Male dispersal likely accounts for these differences. Yearling males may be dispersing from Estes Valley to Middle Park based on this evidence but the incidence of CWD in this age class is low minimizing the risk of CWD transmission. No microsatellite genotypes associated with CWD are evident from our research, however, we were able to support previous findings that gene there were more CWD-positive male deer observed with the S/S genotype and fewer observed with the S/F genotype than expected confirming that PrP genotype at codon 225 was not independent of CWD infection. We also found evidence of increased incidence of CWD in related females which is likely due to similar opportunities for exposure and transmission within matrilineal groups.

## ACKNOWLEDGEMENTS

We would like to thank Margaret Wild and Jenny Powers from the National Park Service Biological Resource Management Division whose training and oversight made deer capture in RMNP possible as well as Vicki Jameson for logistical support. We also thank the RMNP technicians for their deer capture work including Jack Bucklin, Celine Pliessnig, Scott Ratchford, Ryan Rumelhart, Dan Grove, Sam Berry, Shylo Johnson, Marcus Martin, J.P. Farrar, Bill Delanis, Elizabeth Rief and Erin Kunish. Staff from the Colorado Division of Wildlife provided genetic samples as well as technical expertise and we especially thank Mike Miller, Michael Sirochman, Tom Baker, Andy Holland, Justin Martens, Karen Griffin and Laurie Baeten. Chronic wasting disease diagnosis

would not be possible without Terry Spraker and Elaine Andersen from the Colorado State University Veterinary Diagnostic Laboratory. We also acknowledge our collaborators at UCLA especially Hannah Shofi. I thank my graduate committee including Mike Antolin and Tom Hobbs for their mentoring and helpful comments as well as RMNP for funding for the microsatellite genotyping for this project and providing samples and other logistical support.

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**Figure 1: Capture Locations of Sampled Mule Deer**

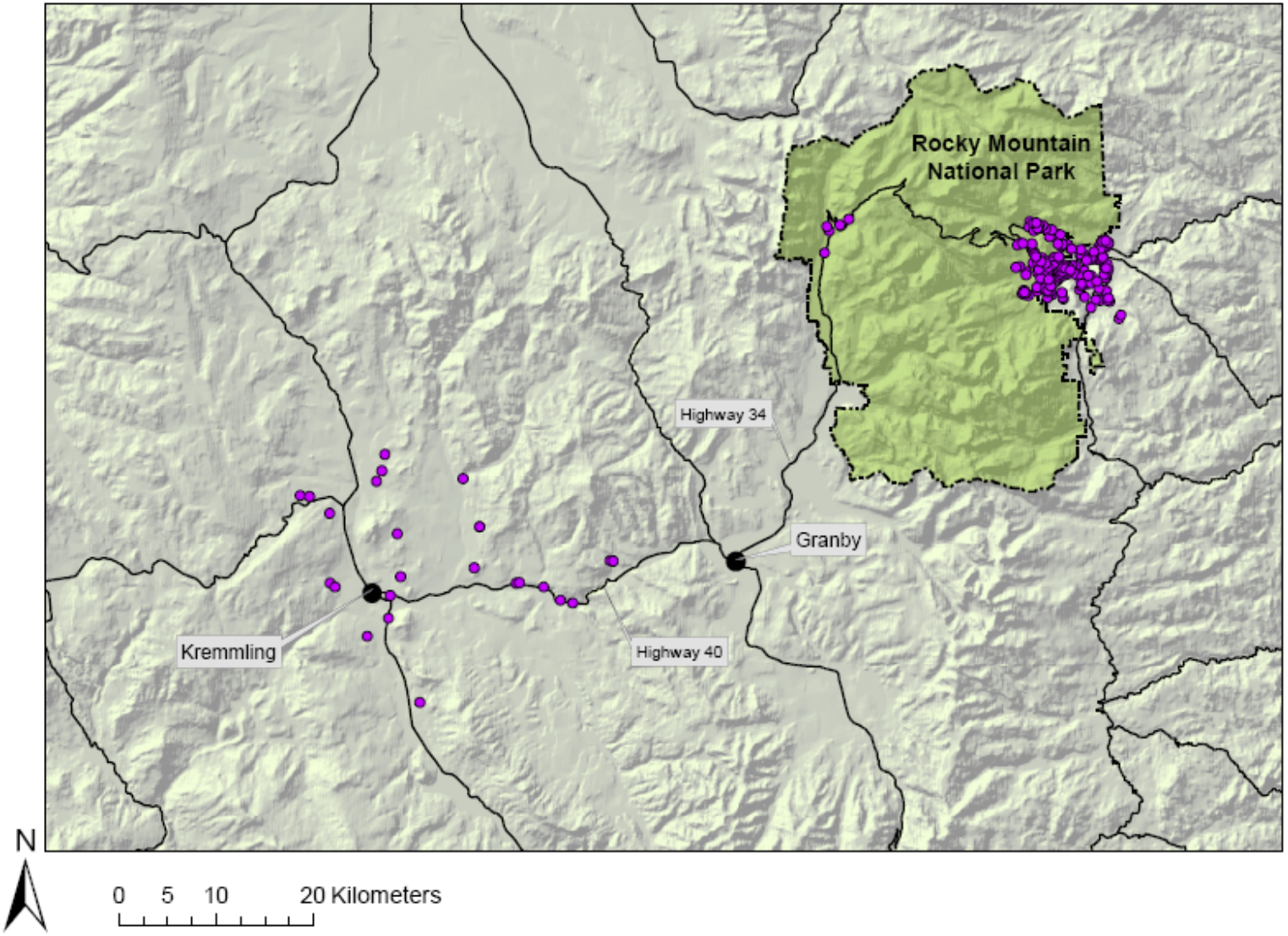

**Table 1: Genetic diversity of microsatellite markers used in genetic analyses**

| | Estes Valley (n=220) | | | | West RMNP (n=5) | | | | Middle Park (n=25) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heterozygosity | | | | Heterozygosity | | | | Heterozygosity | | |
| Locus | Alleles/ locus | Observed | Expected | HWE (*P*-value) | Alleles/ locus | Observed | Expected | HWE (*P*-value) | Alleles/ locus | Observed | Expected | HWE (*P*-value) |
| CI | 6 | 0.619 | 0.617 | 0.97 (S.E.=0.0004) | 3 | 0.400 | 0.340 | 1.0 (S.E.=0.000) | 3 | 0.640 | 0.575 | 0.32 (S.E.=0.004) |
| T7 | 6 | 0.498 | 0.475 | 0.91 (S.E.=0.0008) | 2 | 0.400 | 0.320 | 1.0 (S.E.=0.000) | 5 | 0.520 | 0.618 | 0.32 (S.E.=0.0010) |
| C96 | 4 | 0.405 | 0.434 | 0.64 (S.E. 0.0010) | 3 | 1.000 | 0.580 | 0.17 (S.E.=0.0004) | 3 | 0.480 | 0.465 | 0.67 (S.E.=0.0004) |
| T27 | 12 | 0.848 | 0.874 | 0.022 (S.E.=0.0007) | 6 | 1.000 | 0.760 | 0.80 (S.E.=0.0011) | 9 | 0.818 | 0.846 | 0.83 (S.E.=0.0013) |
| C217 | 6 | 0.696 | 0.730 | 0.30 (S.E.=0.0017) | 5 | 0.800 | 0.740 | 0.90 (S.E.=0.0006) | 5 | 0.773 | 0.652 | 0.50 (S.E.=0.0011) |
| T56 | 9 | 0.688 | 0.757 | 0.10 (S.E.=0.0019) | 5 | 0.600 | 0.740 | 0.022 (S.E.=0.0004) | 8 | 0.720 | 0.792 | 0.53 (S.E.=0.0018) |
| T106 | 6 | 0.592 | 0.649 | 0.03 (S.E.=0.0005) | 2 | 0.800 | 0.480 | 0.43 (S.E.=0.0002) | 6 | 0.522 | 0.615 | 0.12 (S.E.=0.0011) |
| C147 | 3 | 0.588 | 0.565 | 0.79 (S.E.=0.0005) | 2 | 0.400 | 0.480 | 1.0 (S.E.=0.000) | 3 | 0.579 | 0.643 | 0.24 (S.E.=0.0004) |
| Average | 6.5 | 0.617 | 0.638 | 0.11 | 3.5 | 0.675 | 0.555 | 0.64 | 5.25 | 0.632 | 0.651 | 0.49 |

**Table 2. Multilocus $F_{ST}$ by sampling area and sex.**

| Populations compared | $F_{ST}$ | S.E. | *P*-value |
|---|---|---|---|
| All sampling areas | | | |
| All deer | 0.0092 | 0.003 | 0.499 |
| Females | 0.0083 | 0.0063 | 0.28 |
| Males | 0.0007 | 0.0079 | 0.88 |
| Estes Valley vs. West RMNP | | | |
| All deer | -.0012 | 0.0121 | 0.96 |
| Females | -0.0181 | 0.0099 | 0.40 |
| Males | -0.0363 | 0.0539 | 0.70 |
| Estes Valley vs. Middle Park | | | |
| All deer | 0.0118 | 0.0052 | 0.015 |
| Females | 0.0174 | 0.0085 | 0.043 |
| Males | 0.0046 | 0.0083 | 0.51 |
| West RMNP vs. Middle Park | | | |
| All deer | 0.0138 | 0.0089 | 0.45 |
| Females | -0.0027 | 0.0164 | 0.98 |
| Males | -0.0121 | 0.0587 | 0.999 |

**Table 3. Estes Valley deer genotypes at codon 225 of the PrP gene by CWD status**

| | S/S genotype | | S/F genotype | |
|---|---|---|---|---|
| CWD Status | Observed | Expected | Observed | Expected |
| CWD-positive deer | | | | |
| Both sexes | 28 | 24.764 | 2 | 5.2358 |
| Females | 12 | 12.25 | 2 | 1.75 |
| Males | 16 | 12.444 | 0 | 3.5556 |
| CWD-negative deer | | | | |
| Both sexes | 147 | 150.24 | 35 | 31.764 |
| Females | 79 | 78.75 | 11 | 11.25 |
| Males | 68 | 71.556 | 24 | 20.444 |

**Table 4: Population relatedness means and p-values across groups of non-CWD infected and CWD-infected deer.**

| | CWD-negative | | CWD-positive | |
|---|---|---|---|---|
| Deer Group | mean r-value | *P* | mean r-value | *P* |
| All individuals | -0.002 (S.E.-0.001) | 0.51 | 0.001 (S.E.=0.009) | 0.20 |
| Males only | -0.005 (S.E.=0.003) | 0.33 | -0.017 (S.E.=0.014) | 0.98 |
| Females only | 0.005 (S.E.=0.003) | 0.92 | 0.018 (S.E.=0.024) | 0.027 |