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Mitochondrial DNA variation and genetic population structure in Rocky Mountain bighorn sheep (Ovis canadensis canadensis)

Gordon Luikart
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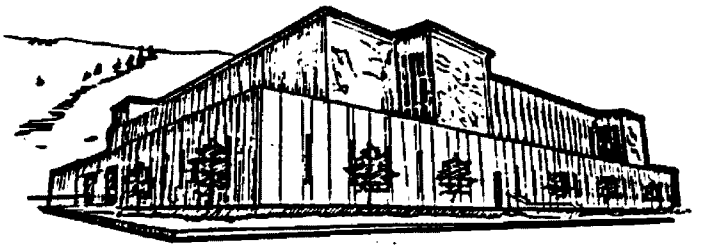

MITOCHONDRIAL DNA VARIATION AND GENETIC POPULATION STRUCTURE IN ROCKY MOUNTAIN BIGHORN SHEEP (*Ovis canadensis canadensis*)

By
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Mitochondrial DNA Variation and Genetic Population Structure of Rocky Mountain Bighorn Sheep (Ovis canadensis canadensis) (67 pp.)

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Bighorn sheep recently have suffered severe reduction and fragmentation of their historical range, and thus have a population structure that renders them susceptible to rapid loss of genetic variation. Transplanting has been and will continue to be used for maintaining viable populations of bighorn sheep. Supplemental transplanting can prevent loss of genetic variation and inbreeding within small herds. However, transplanting individuals between genetically differentiated groups can reduce local adaptation. Understanding the genetic population structure (i.e., the distribution of genetic variation within and among herds) of bighorn sheep would help design transplanting strategies that prevent loss of genetic variation within herds without compromising local adaptations.

The amount and distribution pattern of mitochondrial DNA (mtDNA) variation in Rocky Mountain bighorn sheep was assessed to determine relationships among populations and the usefulness of mtDNA analysis as a tool for conserving genetic variation in bighorn sheep. Restriction enzyme analysis was conducted on mtDNA from over 292 individuals from 22 indigenous herds located throughout the entire geographic range of the Rocky Mountain subspecies of bighorn sheep. Eleven different mtDNA genotypes were identified. A phylogeographic analysis revealed relatively divergent (P=0.78%) mtDNA genotypes within herds, and several geographically widespread genotypes. This suggested that gene flow has occurred on a regional scale at some time in the past (perhaps during colonization of the Rocky Mountains) and that populations have not been subdivided by long-term zoogeographic barriers. Significant differentiation in mtDNA genotype frequencies among herds and among 6 regional groups of herds suggested that little gene flow exists among current populations. The differentiation may be due to population fragmentation and bottlenecks during the last 200 years. Alternatively, because significant genotype frequency heterogeneity exists on a microgeographic scale, much of the observed substructuring could be due to philopatric behavior. Philopatry and population substructure may have existed for thousands of years. The evidence of strong population substructure from this study, and the recent fragmentation of bighorn sheep populations suggest a need for additional studies (e.g., nuclear DNA analyses) of population structure in bighorn sheep.

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INTRODUCTION

Populations in many species are subdivided into local mating groups (demes) as a result of geographic, ecological, or behavioral factors. The genetic population structure of a species is the distribution pattern of genetic variation within and among local demes (Wright 1969). This pattern influences the evolutionary potential of a species and, thus, it affects the probability of a species extinction. For example, species consisting of small, highly divergent demes (lacking interdemic gene flow) suffer accelerated loss of their genetic variation as a result of random genetic drift (random change in allele frequencies and extinction of alleles) and inbreeding effects (Wright 1969, Frankle and Soule 1981).

The Problem:

Many species are suffering accelerated losses of genetic variation as a result of decreased deme sizes and reduced gene flow among demes which has resulted from widespread habitat fragmentation and destruction. Genetic variation is essential for adaptation to future environmental changes and there is growing evidence that genetic variation is also important for maintaining reproductive fitness and viability in present environments. Loss of genetic variation, whether as a result of mating between relatives or population fragmentation, typically results in inbreeding depression

which may consist of increased infant mortality, decreased growth rate and developmental stability, and increased susceptibility to infectious diseases (Ralls et al. 1988; Ralls and Ballou 1983; Schonewald-Cox et al. 1983; Frankel and Soule 1981; Falconer 1981; Leary et al. 1985; Leary et al. 1992).

Ralls et al. (1988) estimated the costs of inbreeding in 38 mammalian species from populations of wild caught and wild caught-captive born individuals. The average cost of full-sibling and parent-offspring matings was a 33% increase in offspring mortality. Fifteen of 16 ungulate species (Eld's deer, *Cervus eldi thamin;* Pere David's deer, *Elaphuris davidianus;* wildebeest, *Connochaetes taurinus;* dik-dik, *Madoqua kirki;* Dorcas gazelle, *Gazella dorcas;* Reindeer, *Rangifer tarandus;* etc.) studied by Ralls and Ballou (1983) exhibited significantly higher juvenile mortality in inbred than non-inbred young.

Mitton et al. (1984) reviewed the association between heterozygosity and growth rate within natural plant and animal populations and concluded that "in virtually every case, the most highly heterozygous individuals enjoy some advantage." Pemberton et al. (1988) found significant associations between survival and heterozygosity at two of the three protein loci examined in red deer (*Cervus elaphus*). In Wyoming, Fitzsimmons (1992) presented evidence that

genetic variability influences horn growth in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*).

Low genetic variation and the loss of it is often associated with high susceptibility to infectious disease. African cheetahs (*Acinonyx jubatus*) have almost no genetic variation, as measured by allozymes, other proteins and major histocompatibility loci (O'Brien et al. 1985, 1986 and 1988). Cheetahs are extremely susceptible to infectious diseases such as feline coronavirus and infectious peritonitis. Cheetah populations may experience 50-100% mortality from these viruses while related felines seldom experience morbidity above 5-10% (O'Brien et al. 1985 and 1988). The black-footed ferret (*Mustella nigripes*) has low nuclear-encoded protein variability and in 1984 a canine distemper epizootic threatened the existence of the species (Thorne and Williams 1988).

Bighorn sheep or mountain sheep (*Ovis canadensis*) have experienced a severe reduction and fragmentation of their historical range (Figure 1). Bighorn sheep have plummeted from over one million individuals in the 1800's to less than 30,000 in the mid-1900's (Buechner 1960; Hoefs 1975). Many populations have become smaller and more isolated, resulting in less gene flow in the species as a whole. Consequently, bighorn sheep have a population structure that renders them susceptible to accelerated decay of genetic diversity.

Bighorn sheep have been reintroduced to much of their former range; However, many reintroductions have not been successful (Cook et al. 1990; Bailey 1990). Cook et al. (1990) evaluated the potential causes of unsuccessful reintroductions of bighorn sheep in Wyoming. They reported that reintroduced herds rapidly increased during the first 6-10 years, but subsequently declined and suffered high lamb mortality and susceptibility to disease. Most reintroduced herds have been founded with few individuals (i.e., less than 30). One factor contributing to post-reintroduction population declines could be increased genetic homozygosity. As mentioned, juvenile mortality and susceptibility to disease are expected effects of inbreeding. Also, if inbreeding depression effects appear, they are expected to do so one to two generations (i.e., approximately 6-10 years in bighorn sheep) after population bottlenecks or founding events (Crow and Kimura 1970).

Thorne et al. (1985) estimated that 64% of 166 herds of Rocky Mountain bighorn (*O. c. canadensis*) and California bighorn sheep (*O. c. californiana*) have fewer than 100 individuals. Berger (1990) studied historical records of 122 desert bighorn sheep populations from the southwest United States and found a positive relationship between population size and population persistence time. Berger showed that all populations with less than 50 individuals became extinct in less than 50 years.

In small herds, inbreeding effects and loss of genetic variation may occur especially rapidly because of the polygynous mating system of bighorn sheep (Geist 1971, Hogg 1984 and 1988). Inbreeding avoidance behaviors can prevent inbreeding that results from mating between close relatives (Ralls et al. 1986). Inbreeding avoidance behaviors may exist in bighorn sheep. However, inbreeding avoidance has never been demonstrated in bighorn sheep and males tend to force copulations with any and all available females (Hogg 1984 and 1988, personal observation). Even if inbreeding avoidance behaviors exist they may be ineffective in small or isolated herds in which mate choice is limited (Hogg pers. comm.).

It has been argued that because mountain sheep have a polygynous mating system, they may be adapted to inbreeding or increased homozygosity (Bailey 1990). However, the only available study that directly assesses inbreeding in bighorn sheep suggests otherwise. Sausman (1984) reported that in captivity, inbred bighorn lambs suffer an average of 34% higher juvenile mortality than non-inbred lambs. This result suggests that mountain sheep evolution has not been characterized by sufficient inbreeding to eliminate deleterious recessive alleles (Templeton 1987). One mechanism causing inbreeding depression is the convergence of deleterious recessive alleles within an individual. Homozygotes for deleterious recessive alleles have reduced

fitness. For example, MHC (major histocompatibility complex) homozygosity has major deleterious influences on successful mating and reproduction in mice, cattle, pigs, horses and chickens (Briles and Allen 1961; Warner 1986; Warner et al. 1987).

Even if deleterious recessive alleles are rare as a result of a history of inbreeding, population subdivision can result in an irreversible loss of alleles from populations (as well as species). Loss of alleles may be especially problematic for gene loci having many alleles such as the MHC loci. MHC genes are involved in disease resistance. Bighorn sheep are extremely susceptible to infectious disease (Festa-Bianchet 1988; Spraker 1979; Wishart 1978; Geist 1971; Potts 1937). Severe population die-offs (i.e., 30-100% mortality) are common and often reoccur cyclically in bighorn sheep populations. Die-offs are probably not a result of loss of genetic variation because bighorn sheep probably did not coevolve with the problematic pathogens (Ramey 1992; M. Dunbar pers. comm.). However, loss of genetic variation as a result of population fragmentation and frequent die-offs may hinder or even preclude the evolution of resistance to rapidly evolving pathogens. Bighorn sheep populations probably cannot afford the loss of adaptive potential that results from population fragmentation and repeated die-offs.

A Solution:

Loss of genetic variation in small, isolated populations can be prevented by transplanting a small number of individuals every few generations (Schonewald-Cox et al. 1983; Allendorf 1983, Varvio et al. 1986). When founding new herds, more than 20-30 individuals should be reintroduced (perhaps in repeated transplants). Transplanting will remain an important management tool long into the future for the following reasons: bighorn sheep are still being reintroduced into their former ranges; populations frequently suffer severe die-offs and require supplementation; and bighorn sheep are relatively easy to capture and transport. Information on the genetic structure (i.e., genetic relationships among local and regional populations) will be useful in making informed transplant decisions. For example, herds with high (or low) genetic variation could be identified and favored (or disfavored) for transplanting.

Genetic population structure analyses can also identify genetically divergent groups that may be locally adapted and that should not be mixed. Bighorn sheep management practices from 1921 to 1990 transplanted 7838 individuals in 552 translocations (Ramey in prep.). Eighty-one translocations have been conducted between geographically distant locations (i.e., between states and provinces). Although transplanting is an option in combatting inbreeding depression, it is not always the best solution. If herds are genetically

differentiated or locally adapted, transplanting can have deleterious effects. For example, in Czechoslovakia a local mountain ibex herd (*Capra ibex ibex*) was supplemented with ibex from other subspecies (*C. i. aegagrus* and *C. i. nuiana*). Hybridization between subspecies altered the rutting time of the local herd so that kids were born in mid-winter. The ibex herd soon became extinct (Templeton 1986). Evidence for local adaptation of lambing time in desert bighorn sheep was reported by Wehausen (1991). Genetic differentiation and local adaptation might exist in herds that have frequently experience epizootics. Also, high elevation and low elevation herds may be differentiated as might northern herds (e.g., Canadian) and southern herds (e.g., Colorado). Bighorn sheep translocations should proceed with caution because populations may be genetically differentiated and because transplanting can spread infectious diseases.

Background

Two species of wild sheep occur in North America: Dall sheep or 'thinhorn sheep' (*Ovis dalli*) of Alaska, and mountain sheep or 'bighorn sheep' (*Ovis canadensis*) ranging from southwestern Canada to Mexico. There are seven extant subspecies of bighorn sheep: Rocky Mountain bighorn, *O. c. canadensis;* California bighorn, *O. c. california;* and five desert bighorn subspecies, *O. c. mexicana, O. c. nelsoni, O.*

c. texiana, *O. c. weemsi*, and *O. c. cremnobates* (see Ramey in prep. for a revision of desert sheep taxonomy). One subspecies, *O. c. audoboni*, has become extinct (Figure 1). Jones et al. (1973) proposed that "Mountain sheep" be used as the official common name for *Ovis canadensis*. However, I use "bighorn sheep" throughout this paper because it makes for easier reference to the different subspecies (e.g., Rocky Mountain bighorn sheep).

Bighorn sheep are a valuable ecological and economic resource. Large herbivores, including bighorn sheep, affect plant communities in many ways. Extinction of a herbivore population may significantly alter community structure and dynamics (Huntly 1991 and citations therein). The bighorn sheep is one of the most highly esteemed big game species in North America (Wishart 1980). Substantial income is generated from hunting permits, sporting goods sales, guiding services and from attraction of photographers, naturalists and tourists. For example, Montana Department of Fish, Wildlife and Parks (MDFWP) has received approximately $100,000 each of the past eight years from the auction of a single bighorn sheep permit (Bill Thomas, MDFWP, pers. comm.).

Knudsen and Allendorf (1987) reported that genetic variation at protein loci in Montana bighorn sheep was higher than average for large mammals (six polymorphic loci, H = 0.39). Hogg and Knudsen (unpublished data), have found

relatively high protein variation in bighorns from Whisky Mountain, Wyoming (5 polymorphic loci), but much lower variation (1-2 polymorphic loci) in herds from Ram Mountain and Sheep River in Alberta, Canada. Hogg and Knudsen (pers. comm.) recently discovered significantly different frequencies of allozymes between sympatric herds within the Whisky Mountain area. This finding suggests that microgeographic subdivision as a result of behavioral mechanisms may exist among bighorn herds. Because only one polymorphic protein locus (transferrin, two alleles) has been detected in some populations of Rocky Mountain bighorn sheep, protein electrophoresis cannot detect enough variation to thoroughly assess genetic population structure.

Hogg et al. are continuing protein analyses and starting nuclear DNA surveys as part of an ongoing comprehensive study to: 1) estimate effective population size from behavioral, demographic and genetic data; and 2) evaluate the importance of genetic variation to reproductive fitness and general vigor. Ramey (1991b) is employing mtDNA restriction enzyme analysis and sequencing of the D-loop to determine the phylogenetic relationships of North American wild sheep. Ramey (pers. comm.) may also assess population structure within the putative desert subspecies of bighorn sheep using mtDNA analysis.

Several characteristics of mtDNA make it useful for assessing population structure. There is little or no

indication that mtDNA sequence variation is maintained by natural selection (Avise et al. 1987; Forbes and Allendorf 1991). Consequently, mtDNA is appropriate for population genetic analyses such as estimating population structure, gene flow, and genetic relatedness, all of which require selectively neutral markers (Karl and Avise 1992). Intraspecific mtDNA variation is usually high (Avise and Lansman 1983) because its nucleotide substitution rate is 5-10 times that of nuclear genes (Brown et al. 1979). The effective population size for mtDNA genes is approximately one-fourth that of nuclear genes because mtDNA is maternally inherited (Gyllensten et al. 1985; Lansman et al. 1983b) and because individuals are haploid (Nei and Tajima 1981; Birkey et al. 1983 and 1989). As a result of MtDNA's high mutation rate and small effective population size, mtDNA analyses may resolve population relationships when nuclear genes do not. Finally, because mammalian mtDNA does not undergo recombination (Brown 1983), phylogenetic relationships and phylogeographic analyses of genetic population structure can be conducted relatively easily (Avise et al. 1987).

As a result of mtDNA's maternal inheritance, the pattern of population subdivision revealed by mtDNA analyses (maternal population subdivision) may differ from the pattern revealed by nuclear genes. For example, because only females contribute to mitochondrial gene flow, species in which females disperse less than males may exhibit greater

subdivision for mtDNA than for nuclear genes. However, this greater population subdivision may be lessened or reversed in polygynous species where more females reproduce than males (Birkey et al. 1983 and 1989). Because bighorn sheep are polygynous (Geist 1971, Hogg 1984 and 1988) and females probably disperse less (Ough and deVos 1984; Witham and Smith 1979; Cochran and Linwood 1983; Munoz 1981; and personal observation), population subdivision for mtDNA may not equal the subdivision for nuclear genes.

Recent publications have shown that mtDNA markers provide an informative view of population structure (Avise et al. 1987; Moritz et al. 1987; Wilson et al. 1985). Restriction enzyme analysis of mtDNA has been used to assess phylogeographic population structure in large terrestrial mammals (Cronin et al. 1989, 1991a, 1991c, and In press; Ramey 1991; Lehman and Wayne 1991; Wayne et al. 1990; Wayne et al. 1991; Vigilant et al. 1991), in marine mammals (Hoelzel 1991), in many small mammals (Avise et al. 1987; Yamagata et al. 1990; Phillips et al. 1989; Honeycut et al. 1988), in birds (Moore et al. 1991; Zink et al. 1991; Ovenden et al. 1991, Ball et al. 1988; Shields and Wilson 1987; Tegelstrom et al. 1990), in amphibians and reptiles (Bowen 1991; Bowen et al. 1992; Nichols 1989), in fish (Ryman and Utter 1987), and in many invertebrates (DeSalle et al. 1987; Reeb and Avise 1990; Avise 1987; Brown 1991). MtDNA analyses have also been used to assess population structure

on a microgeographic scale using frequency heterogeneity analyses (Kessler and Avise 1985; Plante 1989; Cronin 1991a; Moore et al. 1991).

Objectives

The broad objective of this research was to assess genetic relationships among populations of Rocky Mountain bighorn sheep using restriction enzyme analysis of mtDNA. More specifically, the objectives were as follows:

1) To estimate the amount of mitochondrial DNA (mtDNA) variation in Rocky Mountain bighorn sheep;
2) To assess the degree and pattern of population differentiation; and
3) To identify herds with low (or high) mtDNA variation and test for loss of mtDNA variation in a reintroduced herd relative to its source herd.

Attainment of the first objective will ascertain the usefulness of mtDNA restriction enzyme analysis as a conservation genetics tool. Attainment of the second and third objectives will help wildlife managers conserve genetic variation within and among herds. With respect to these latter objectives, I hypothesized that population differentiation would be extensive, even on a microgeographic scale, because females exhibit group-cohesive behavior and because the effective population size for mitochondrial DNA

is probably small. It was also hypothesized that mtDNA variation would be significantly lower in the reintroduced herd when compared to its source herd because mtDNA variation is lost rapidly during population bottlenecks.

MATERIALS AND METHODS

Study Herds and Collection of Samples:

Samples were collected from 292 individuals and 22 indigenous herds located throughout the entire geographic range of Rocky Mountain bighorn sheep (Figures 2 and 4). None of these herds are known to have received transplanted bighorn sheep. In addition, 19 samples from a transplanted herds (Rampart) in Colorado were analyzed. Bighorn sheep were sampled at wildlife check stations or by corral-trapping, drop netting or dart gunning, with the generous help of many state and provincial wildlife agency personnel, veterinarians, and other biologists. The sex, approximate age, and geographic source of each individual was recorded.

From live-captured individuals, 5-10 milliliters of whole blood was collected by venipuncture of the jugular vein. Samples were then dissolved in a heparin or EDTA vacutainer tube and frozen in liquid nitrogen or in a -5 ^{o}C freezer. Clotted blood, from which the serum had been removed, was obtained from the Bureau of Land Management (BLM) herd and all of the Colorado herds. Clot-containing vacutainers were placed in plastic bags, frozen and express-mailed to the

University of Montana. From hunter-killed bighorn sheep, one cubic inch of skeletal muscle or liver tissue was collected 12-48 hours after death. Samples were placed in a sterile, buffered EDTA solution, frozen for 2-8 weeks at approximately -5 °C, and shipped to the University of Montana. Samples were stored for up to one year at -40 °C before the DNA was isolated.

Laboratory Procedure

Restriction endonuclease techniques were used for all mtDNA analyses. Total cellular DNA was isolated from the blood samples using the salt-chloroform procedure of Mullenbach et al. (1989), with the addition of one phenol-cholorform extraction. From muscle and liver tissue, CNAs (cytoplasmic nucleic acids) were isolated by the rapid technique described by Lansman et al. (1981). After digestion, restriction fragments were separated by electrophoresis on 0.8-2.0% agarose gels (Maniatis et al. 1982). Electrophoresed fragments were then denatured, and transferred to nylon filters by alkaline blotting (Southern 1975). Filters were hybridized in 2 x SSC over night at 60 °C with radioactively (^{32}P) labeled probe. MtDNA fragment patterns were visualized using autoradiography (Maniatis et al. 1982). Highly purified mtDNA for use as probe was prepared from fresh domestic sheep liver by CsCl-ethidium bromide gradient methods (Lansman et al. 1981). Cloned mule

deer mtDNA, kindly provided by M. Cronin and E. Vyse, was also used as probe. Mule deer probe revealed fragment patterns identical to those detected by the domestic sheep probe (See Cronin, 1989, for information on mule deer mtDNA cloning and its use for probing many ungulate species' mtDNA.) Nonradioactive probe using Boehringer Mannheim Biochemical's 'Genius Labeling and Detection Kit' was also used. The mobility of fragments was compared to molecular weight/size standards of known measurement provided by Hind III digests of lambda DNA. Each unique fragment pattern produced by a given restriction enzyme was identified and labeled with an upper-case letter. The most common pattern for each enzyme was arbitrarily labeled "A". Different combinations of upper case letters represent different composite mtDNA genotypes (haplotypes, clones, lineages) (Table 2).

Data analysis

A phylogeny of mtDNA haplotypes was generated from fragment pattern data using cladistic parsimony and the Branch and Bound option of the PAUP computer program (Swofford 1991). Parsimony analysis is especially appropriate when mtDNA genotypes differ by one or few mutational transformations, and if divergences are less than 1.0% (Nei and Tajima 1985). The Branch and Bound option is exhaustive (evaluates all possible trees) and, thus,

guarantees finding the most parsimonious tree(s). Alternate fragment patterns (e.g., patterns "A", "B" and "C") identified by one restriction enzyme were treated as alternate (ancestral or derived) and unordered character states. One Dall sheep (*Ovis dalli dalli*) from the Brook's Range, Alaska, was assayed for use as an out-group to root the phylogeny. Phylogeographic population structure was assessed qualitatively (as in Avise et al. 1987) by evaluating the degree of concordance between phylogeny and geography. For example, the following questions were addressed: Are there major phylogenetic discontinuities associated with a long-term zoogeographic barrier to gene flow? Are haplotypes widely distributed geographically? Avise et al. (1987) provide a detailed description of the possible categories of phylogeographic population structuring.

Frequency heterogeneity tests for population differentiation included Wright's hierachial F-statistic analysis using the STEP WRIGHT78 option (corrected for haploid data) in the BIOSIS computer package (Swofford and Selander 1989), UPGMA clustering of populations based on similarity of haplotype frequency as calculated by Nei's unbiased genetic identity (BIOSIS, CLUSTER option), and contingency table Chi-square analysis via Roff and Bentzen's (1989) Monte Carlo resampling procedure which corrects for small sample sizes. The significance level ($P < 0.05$) for

multiple tests was reduced according to the number of tests conducted (e.g., $P < 0.05/n$, where n=number of tests).

Haplotype diversity (h) was estimated to compare the amount of mtDNA variation in different subpopulations and regions, and to compare a reintroduced herd with its source herd. Haplotype diversity is equivalent to heterozygosity used for measuring protein variation, and is defined as the probability that two randomly sampled mtDNAs will be of different types (alleles). Haplotype diversity was calculated according to the Birkey et al. (1989) equation:

$$\hat{h} = 1/n-1 \; [\; n \; (1- \; \Sigma \; x_i^2) \;]$$

where 'x_i' is the frequency of the 'ith' haplotype, and 'n' is the sample size.

RESULTS

In a pilot survey, a single individual from each of 7 geographically distant herds was cleaved with each of 23 restriction endonucleases (enzymes). This survey revealed 17 informative restriction enzymes; that is, enzymes that cleaved *O. c. canadensis* mtDNA at two or more sites and, thus, provided information on nucleotide sequence diversity. The 17 enzymes consisted of fourteen 6-base cutters; one 5-base cutter; and two 4-base cutters (Tables 1 and 2).

The seventeen enzymes (Table 1 and 2) cleaved Rocky Mountain sheep mtDNA at an average of 113 restriction sites resulting in a 2.8% sample of the mtDNA genome. With the

exception of some Hinf-I restriction-fragment patterns, mutational transformations between variant restriction-fragment patterns were easily discernable as single restriction-site losses (or gains). By summing the lengths of fragments produced by 6-base cutters, the size of the mtDNA genome was estimated to be 16.3 kilobases in length. This length is similar to that of the desert sheep mtDNA genome (Ramey 1991), and similar to that of other ungulates (Cronin 1989). Of the 17 enzymes employed, only 7 detected fragment pattern polymorphisms in Rocky Mountain sheep (Table 2). These fragment pattern polymorphisms revealed 11 distinct mtDNA-genotypes (clones, lineages or haplotypes) (Table 3).

Sequence Divergence and Haplotype Richness

Thirty-nine individuals from geographically distant locations were analyzed with the 17 informative restriction enzymes to estimate percent sequence divergence (nucleotide substitutions per base-pair multiplied by 100) (Table 4). Nucleotide sequence divergence estimates were calculated using the "fragment data" approach and Nei and Li's (1979) equation 20. The average percent sequence divergence among all pair-wise comparisons of the 11 Rocky Mountain bighorn sheep mtDNA haplotypes was 0.43%. The range was 0.1% through 0.78%. MtDNA variation (measured as haplotype richness and as sequence divergence) in Rocky Mountain sheep is low

relative to most mammals (Avise and Lansman 1983), moderatly-high relative to five other species of North American ungulates (Cronin 1989), and high in comparison to desert bighorn sheep (Ramey 1991) (Table 4). Significantly, enough mtDNA variation exists to allow at least a preliminary assessment of genetic population structure using restriction enzyme techniques.

Genetic Population Structure

Two approaches were used to assess genetic population structure: a systematics approach called "Intraspecific Phylogeography", and a traditional population genetics approach involving tests for heterogeneity of haplotype frequencies.

Intraspecific Phylogeography: Intraspecific Phylogeography is the study of the concordance between phylogeny (relationships among haplotypes or alleles) and geography (Avise et al. 1987). Intraspecific phylogeography presupposes that if there has been little or no gene flow among geographic areas, haplotypes in different areas will be more divergent than haplotypes in the same area. This will result in concordance between phylogenies of haplotypes and the geographic distribution of haplotypes (Slatkin 1989).

Cladistic parsimony analysis (PAUP, Swofford 1992) of 11 Rocky Mountain bighorn sheep haplotypes resulted in 174 equally parsimonious trees of 23 mutational steps in length.

The 174 parsimony trees were summarized by a strict consensus tree (consistency index = 0.92) which appears in Figure 3. Although the consensus tree (or network) is only one of many phylogenies that could explain the relationships among haplotypes, no trees revealed major phylogenetic discontinuities (phylogenetic breaks). That is to say, divergence is relatively low (1-2 mutational steps) between haplotypes that are adjacent in the phylogeny.

Sixty-three individuals were analyzed with enough diagnostic enzymes for use in the phylogeographic assessment of population structure (Table 3 and 4). Several haplotypes (#1, #2, #6, and #8) occur over a wide geographic area, and substantially divergent haplotypes (#10 and #1) occur together within the same herds (Figure 3). These observations suggest that little phylogeographic population structure exists and that gene flow has occurred on a regional scale in the past. Preliminary evidence of geographic localization for some divergent haplotypes (e.g., haplotypes #4 and #5 in central Montana, and #10 and #11 in Colorado), however, suggests that gene flow may have been limited more recently. This type of evidence of localization is preliminary and inconclusive because rare haplotypes (Table 3) may not have been detected at some locations with small sample sizes.

Haplotype frequency heterogeneity: Haplotype frequency heterogeneity analyses were conducted to examine regional and

local population differences more rigorously. Frequency heterogeneity analyses are more sensitive to recent population structure than phylogeographic analyses because frequency differentiation can develop without thousands of years of population isolation.

Frequency heterogeneity analyses require large sample sizes. Large sample sizes have been difficult to obtain because of the time and expense of restriction enzyme analysis (Roff and Bentzen 1989). However, using a few diagnostic restriction enzymes on many individuals can provide large sample sizes (DeSalle et al. 1987; Cronin 1991a). In this frequency heterogeneity assessment of population structure, 292 total samples were cleaved with 3 diagnostic enzymes that consistently and unambiguously cleaved mtDNA. These 3 restriction enzymes (Hae III, Sty I, and Bam HI) identified 5 haplotypes (Table 3 and Figure 4), a subset of the 11 haplotypes used in intraspecific phylogeography.

To increase sample sizes within subpopulations, adjacent subpopulations that had small sample sizes were pooled. Subpopulations were pooled within each of five geographic areas: British Columbia; southern Montana (Spanish Peaks, Tom Minor, and Gardiner); Idaho (Salmon River) and western Montana (Bitterroot River); and the North and South Forks of the Shoshone River in Wyoming (Figures 2 and 4). Fifteen subpopulations were used in frequency heterogeneity analyses.

As Figure 4 shows, differences in the frequency of the five haplotypes are common on a local and regional scale.

To test for frequency heterogeneity on a regional scale, subpopulations were grouped according to their geographical proximity. Groups consisted of Canada, the Sun River area, Idaho/W. Fork Bitterroot River, southern Montana, Wyoming, and Colorado (Figure 4). The six groups appear to be differentiated in the following ways: the Canadian herds contain almost exclusively haplotype 'AAA'; the Sun River herds have a high frequency of the otherwise rare haplotype 'ABA'; the Wyoming herds contain almost exclusively haplotype 'BAA'; and the Tarryall, Colorado, herd is the only indigenous herd in which haplotype 'BAB' was detected.

Wright's hierarchial F-statistic analysis was used to partition total variance of haplotype frequency into two components: variation among all subpopulations and variance among regional groups of subpopulations. The F-statistic analysis and the contingency Chi-square tests revealed substantial haplotype frequency differentiation among the 15 subpopulations ($F_{st} = 0.62$; $P < 0.001$ for the Chi-square test), and among the 6 groups of subpopulations ($F_{gt}=0.41$; $P < 0.001$ for the Chi-square test).

UPGMA clustering grouped herds according to similarity in haplotype frequencies. Clustering revealed two highly differentiated, yet geographically interspersed, groups of herds (Figure 5). Herds in one group are characterized by a

high frequency of the 'AAA' haplotype (black in the pie graphs, Figure 4), while herds in the other group are characterized by relatively high frequency of the 'BAA' haplotype (white in the pie graphs). Each of the two groups contain herds from throughout much of the geographic range of Rocky Mountain sheep, suggesting that no macrogeographic pattern of population differentiation exists.

Genetic differentiation on a finer geographic scale is indicated by the high value of the F-statistic that measures differentiation among subpopulations within regional groups (Fsg = 0.36). Genetic differentiation on a microgeographic scale was further examined by comparing contiguous herds. Although the four Sun River herds (Deep Creek, Gibson Reservoir, Castle Reef, and Ford Creek) are within 5-15 kilometers of each other, movement studies (Erickson 1972; Fisina 1974) have shown that ewes, and perhaps even the rams, maintain cohesive groups. A contingency table test detected significant heterogeneity ($P < 0.001$) in haplotype frequencies among the four herds. The two largest Sun River herds, Castle Reef, and Gibson Reservoir, rut in areas that are separated by only five kilometers. These two herds are not significantly differentiated for mtDNA genotypes. The two Whiskey Basin herds, Torry Rim and BLM, maintain cohesive groups with little female dispersal between them (Thorne et al. 1979), yet they are separated by less that eight kilometers on both their summer and winter ranges. No

differences in haplotype frequencies between the two groups were detected; However, too little mtDNA variation was present in the herds for informative tests of frequency heterogeneity.

Contingency table tests were conducted for differences between two sampling techniques used in the Gibson Reservoir and Castle Reef subpopulations. Samples were collected from hunter-killed individuals (sampled in November, 1990) and corral-trapped individuals (sampled in March, 1991) within the Gibson and within the Castle Reef winter/rutting ranges. The hunter-killed individuals were taken from many locations dispersed 1 to 2 miles apart throughout the local winter ranges, whereas coral-trapped individuals were taken from the center of the Gibson Reservoir and Castle Reef winter ranges, respectively. Differences in mtDNA frequencies between the two sample types were not significant within either the Castle Reef ($P = 0.12$) or the Gibson Reservoir ($P = 0.47$) populations. This suggested that samples collected by the two different techniques could be pooled for use in statistical analyses. Similar haplotype frequency estimates from different sampling techniques (and from different times of year) suggest that the frequency estimates are representative of the true subpopulation frequencies.

In addition, haplotype frequencies were not significantly different between males and females sampled within either the Castle Reef or the Gibson Reservoir sites. This suggests

that higher mobility of males is not substantially influencing haplotype frequencies (Birkey et al. 1983) and that the sexes can be pooled for statistical analyses.

Haplotype diversity within populations

MtDNA haplotype diversity was calculated using the frequencies of the five haplotypes listed in Figure 4. Haplotype diversity in *O. c. canadensis* was $\hat{h} = 0.61$. Within populations, haplotype diversity was highest in the Sun River herds of Montana and in the Tarryall herd of Colorado (Table 6). The Canadian and Wyoming herds had the lowest diversity and almost all were fixed for one haplotype. A t-test was conducted (as in Nei 1987, pg. 183) to test for reduced haplotype diversity in a reintroduced herd, Rampart, relative to its source herd, Tarryall. Rampart was founded with only 14 individuals in 1946 and supplemented with 20 in 1984. Rampart had significantly lower haplotype diversity ($P<0.005$) than its source herd, Tarryall.

DISCUSSION

Sequence divergence and Haplotype Richness

Both measures of mtDNA variation (haplotype richness and sequence divergence) in Rocky Mountain sheep were above the average for North American ungulates. The amount of mtDNA variation in Rocky Mountain sheep (11 haplotypes and 0.78% maximum sequence divergence) is higher than the mtDNA

variation among several groups of desert sheep. Only 4 mtDNA haplotypes and less that 0.20% sequence divergence was detected among 31 desert bighorn sheep that included representatives from 3 desert sheep subspecies (*O. c. nelsoni, O. c. cremnobates, and O. c. mexicana*) (Ramey 1991). Higher mtDNA variation in Rocky Mountain bighorns is not surprising in light of studies of nuclear-encoded protein variation which also revealed much higher genetic variation in Rocky mountain sheep than in the desert sheep groups (Knudsen and Allendorf 1987; Ramey 1991; Wraxall and Dehaan 1985).

Ramey's studies (1991, in prep.) of mountain sheep and this study of Rocky Mountain bighorns used nearly identical sets of restriction enzymes and, consequently, are more similar than most studies. However, because estimates of sequence divergence and haplotype richness are dependent on sample size, geographic range of sampling, and the number of restriction enzymes used, comparisons between studies are difficult. A measure of variation less affected by sample size is gene diversity or haplotype diversity (Chakraborty 1991). Unfortunately, few studies report this measure.

Even though it is difficult to compare between studies, mtDNA variation is so much higher in Rocky Mountain bighorn sheep than in elk (*Cervus canadensis)* and moose (*Alces alces*) (Table 5) that the difference is undoubtedly significant--especially when one considers that the geographic range

sampled was far greater in the elk and moose studies than in this study. Higher variation in Rocky Mountain sheep is somewhat surprising in light of historical and current geographical distributions of these species. Mountain sheep, elk, and moose were all Pleistocene immigrants to North America. Elk and moose probably existed north and south of the Pleistocene glacial sheets (Cronin 1991), whereas Rocky Mountain sheep were confined south of the ice sheets during the Pleistocene glaciations (Geist 1985). Rocky Mountain sheep currently have a much smaller geographic range than elk and moose which are holarctic in distribution (Cronin 1991). These observations would seem to predict less mtDNA variation in the Rocky Mountain sheep subspecies than in elk and moose species.

One explanation for the relatively high mtDNA variation (Pmax = 0.78%) in contemporary Rocky Mountain sheep populations could be a higher rate of mtDNA evolution. If the rate of mtDNA sequence divergence in bighorn sheep is approximately 2% per million years as it is in other vertebrates (Wilson et al. 1985), then the most recent common female ancestor of all contemporary Rocky Mountain sheep (given Pmax = 0.78%) lived approximately 390,000 years ago. This is in close agreement with the age of the oldest known bighorn sheep fossil which is 300,000 years old (Geist 1985). The relatively high mtDNA variation in Rocky Mountain sheep is thus probably not due to a molecular clock "ticking" at a rate much faster than 2% per million years.

An alternative explanation for the relatively high mtDNA variation is that divergent mtDNA lineages have been maintained in mountain sheep while genetic drift has caused more rapid lineage extinction in elk and moose. In mountain sheep, maintenance of divergent haplotypes could result from relatively strong population substructure (i.e., less gene flow) in comparison to elk and moose.

Genetic population structure

Haplotype frequency heterogeneity: The significant among-population heterogeneity in haplotype frequency, and the finding that many populations are fixed or nearly fixed for one haplotype, show that contemporary Rocky Mountain sheep populations are highly differentiated (i.e., substructured) for mtDNA variation. F-statistics (measures of population differentiation) for Rocky Mountain sheep are high (Fst = 0.62, Fsg = 0.36) relative to πthe only similar studies in ungulates, Montana mule deer (Fst = 0.20) and Minnesota white-tailed deer (Fst = 0.05) (Cronin 1991a). The significant haplotype frequency heterogeneity among regional groups of herds, and the predominance of different haplotypes in different geographical regions, suggest that regional populations are differentiated for mtDNA.

The population differentiation is probably a result of strong stochastic lineage sorting (genetic drift) within

herds, and low mtDNA gene flow among contemporary herds. Substantial genetic drift is expected for several reasons: bighorn sheep have overlapping generations; few breeding females exist in most populations (e.g., $N < 100$); herds experience substantial changes in population size through time; and variation in female reproductive success may be high. Festa-Bianchet (pers. comm.) has found high variation in reproductive success among ewes in the Sheep River, Alberta, herd.

Low mtDNA gene flow among populations may be a result of habitat fragmentation and population bottlenecks during the past 200 years. However, gene flow among populations may also have been limited long before the recent fragmentation. Because significant haplotype frequency differentiation occurs on a microgeographic scale, it is possible that much of the observed population differentiation and lack of gene flow is a result of philopatric, group-cohesive behavior. Strong group-fidelity has been observed in movement studies of Rocky Mountain sheep ewes (Erickson 1972; Fisina 1974; Thorne et al. 1979; pers. comm. Festa-Bianchet). It is possible that philopatry has existed for thousands of years and that much of the population differentiation predates the recent population fragmentation.

For example, females under normal conditions are probably philopatric. However, some females may be predisposed to

disperse as exemplified by observations that small groups occasionally split-off from their maternal group, and wander around the fringe of a neighboring ewe-group's home range (pers. comm. Hogg; personal observation). These wanders usually do not stay or are not accepted into the neighboring group (Erickson 1972; Thorne et al. 1979; pers. comm. Hogg) and thus gene flow (gene-pool mixing) does not occur. However, the opening of geographic areas as a result of local extinction or rapid environmental change (e.g., post-glacial environmental change) would allow dispersal-predisposed females to colonize neighboring habitats and gene flow (spread of genotypes across the landscape) to occur. This scenario, although speculative could explain how mtDNA genotypes have become geographically wide-spread yet populations are strongly differentiated for genotype frequencies, on a regional and microgeographic scale. This scenario is supported by behavioral, dispersal, and mtDNA data (Hogg and Luikart in prep.).

In addition to philopatry, the spread of forests may have reduced interpopulation gene flow long before the recent population fragmentation. After the last Pleistocene glaciers receded (15,000 years ago) and climates changed, forests spread up mountain valleys and between mountain ranges (Geist 1971). Forest and brush encroachment has reduced sheep ranges and cut off migration routes (Cunningham

Intraspecific phylogeography: Phylogeographic analysis can estimate the length of time that populations have been isolated or substructured. For example, finding a phylogenetic discontinuity separating populations that occur in distinct geographic areas would suggest that the populations have been reproductively isolated for a long time (i.e., a length of time proportional to the amount of genetic divergence between populations). As mentioned, the longer populations have been isolated, the greater the likelihood that local adaptations would have developed.

The bighorn sheep mtDNA phylogeny did not reveal phylogenetic discontinuities and, consequently, provided no evidence of long-term population isolation. The lack of major phylogenetic discontinuities in Rocky Mountain sheep is not surprising given their relatively recent (approximately 300,000 years ago) colonization of North America. It appears that populations have not been isolated long enough (i.e., many thousands of generations) for major mtDNA discontinuities to accumulate. Therefore, extensive local adaptations are unlikely. Local adaptations may be unlikely, but they may exist if strong natural selection has occured. For example, repeated population die-offs caused by pathogens could produce strong selection and adaptive differentiation in a short time (i.e., hundreds of generations). Different herds may have recently evolved with different strains of

pathogens and, thus, become adaptively differentiated. Some herds may have never been exposed to certain pathogens. For example, a Pasteurelosis die-off has never been recorded in the Ram Mountain herd in Canada (pers. comm. J. Jorgenson). Additional independent measures of population relatedness (e.g., nuclear DNA, morphometrics etc.) could help determine the likelihood of local adaptation.

The wide geographic distribution of relatively divergent haplotypes #1 and #8 and other haplotypes (#2 and #6) suggests that little or no phylogeograhic population structure exists among Rocky Mountain bighorn sheep herds. Lack of phylogeographic structure (i.e., lack of concordance between phylogeny and geography) suggests that, at some time in the past, gene flow has occured on a regional-scale (i.e., genotypes spread over a wide geographic area). Occurrence of regional-scale gene flow would not be surprising given the high potential for dispersal in mountain sheep.

More samples are needed to determine if haplotypes are geographically localized or if they simply appear localized because of a limited sample size. If additional sampling reveals that the relatively divergent haplotypes (e.g., #4 and #5 in Montana and #10 and #11 in Colorado) are localized, it would suggest that, at least in recent times, gene flow has been limited and/or that genetic drift has been

substantial. The localization of haplotypes would not necessarily contradict the conclusion that gene flow has occurred on a regional scale. For example, the regional spread of haplotypes could have occurred long ago (e.g., during initial colonization or during post-glacial recolonization) and the localization may be a more recent development.

The idea of regional-scale gene flow during colonization is supported by the observation that the most common and widespread haplotype is also the most ancestral (#1 in the phylogeny, or AAA in the frequency analyses). Unfortunately, we cannot determine the date or duration of the gene flow that led to the wide geographic distribution of haplotypes. MtDNA genotypes may not have become widespread during a short period of gene flow (i.e., during colonization); Haplotypes could have spread on a regional scale if low levels of gene flow existed throughout the history of Rocky Mountain sheep.

The overall phylogeographic pattern for Rocky Mountain sheep is one of phylogeographic continuity, with some widespread and some geographically localized haplotypes; However, the sample sizes were too small to determine with certainty the extent of haplotype localization. This overall pattern also occurs in caribou, *Rangifer tarandus*, and deer, *Odocoileus virginianus* (Cronin 1991). Avise et. al. (1987) described five categories of possible phylogeographic

population structures. Rocky Mountain sheep fall in between category #5 and #4 (species with "intermediate or extensive gene flow not subdivided by long-term zoogeographic barriers") Avise et al. (1987). Interestingly, most large, mobile mammals studied to date (coyotes, *Canus latrans,* Lehman and Wayne 1991; African jackals, *Canus aureus,* Wayne et al. 1990; white-tail deer, *Odocoileus virginianus,* elk, *Cervus canadensis,* moose, *Alces alces;* and caribou, *Rangifer tarandus,* Cronin 1991; bears, *Ursus americanus, U. arctos, Thalarctos maritimus,* Cronin In press; and bighorn sheep, this study) have weak or no phylogeographic structuring. In fact, highy divergent mtDNA haplotypes have even been found within local populations. For example, divergent haplotypes have been detected within local populations of African jackals (P = 8.0%; Wayne et al. 1990) and in North American black bear (P = 5.0%; Cronin et al. In press). Not surprisingly, many small, relatively immobile mammals (deer mice, *Peromyscus maniculatus;* pocket gophers, *Geomys bursarius;* white-footed mouse, *Peromyscus leucopus;* Avise et al. 1987) have extensive phylogeographic population structure (i.e., phylogenetic discontinuities associated with zoogeographic barriers).

Haplotype diversity within populations

MtDNA diversity is highest in the Sun River herds of Montana and the Tarryall herd of Colorado (Table 6). High mtDNA diversity is probably indicative of high diversity for nuclear genes because mtDNA variation is generally lost at a faster rate than nuclear gene variation (Birkey et al. 1983 and 1989). High genetic diversity is important in the Sun River and Tarryall herds because they are major source-herds for transplanting. Interestingly, both herds have suffered severe population die-offs. For example, the Tarryall herd was estimated to have been reduced to approximatley 12 individuals during 1923-24. During the 1952-53 die-off, Tarryall and an adjacent herd (Kenosha) plummeted from approximately 950 to only 44 individuals (Buchner 1960). MtDNA diversity may have been maintained during these die-offs by preservation of different lineages in different subpopulations (breeding groups) within the herds. Tarryall has two distinct breeding groups (Mike Miller pers. comm.) which could have preserved different mtDNA lineages during population bottlenecks. The Sun River area has had at least eight different breeding areas (Erickson 1972). Substantial metapopulation structure has probably helped maintain diversity during die-offs in the Sun River herds.

MtDNA diversity is low in the Canadian populations. Fifty individuals were sampled from six Canadian herds. Forty-eight of these individuals had the same "AAA"

haplotype. Allozyme variation is also low in Canadian herds (Hogg et al., unpublished data) in comparison to variation in Montana, Wyoming, and Colorado populations. Reduced genetic variation in Canadian herds may be explained by founder effects and genetic drift. Following the Wisconsonian Ice Age (10,000-20,000 years ago), small bands of mountain sheep may have recolonized Canada. If population sizes remained small, most mtDNA lineages would have been lost as a result of genetic drift. A stepping stone model of recolonization could also explain the low variation in Canadian herds. Fluctuating ice fronts of repeated glaciation cycles may have caused a repeated "stepping stone model of range recolonization" that "produced a serial loss of alleles from the oldest to the youngest populations, as deme founders at the leading edge repeatedly transferred smaller and smaller subsets of the parental alleles to the daughter populations" (Sage and Wolff 1986).

In Wyoming, 75 of the 78 individuals sampled had the same haplotype (BAA). High mtDNA diversity might be expected in northwest Wyoming because it maintains many large, contiguous herds and because the fossil record suggests that Wyoming was a glacial refugium for Rocky Mountain sheep (Geist 1985). Population die-offs in the area, however, may explain the low mtDNA variation. Honess and Frost (1942) reported that in 1940, only 500 sheep were left in the entire Wind River Mountain Range. (The Torry Rim and BLM herds in this study

are in the Wind River Mountains.) Genetic diversity in the BLM and Torry Rim herds is of interest because they are source-herds for many transplants.

The significantly lower mtDNA diversity in the reintroduced herd (Rampart), compared to its founder herd (Tarryall) was not surprising since Rampart was founded with few individuals and remained small (i.e., $N < 60$) for 30-40 years (Bailey 1988). This comparison of a reintroduced herd verses its source-herd suggests that mtDNA analysis may be useful for monitoring within herds for loss of genetic variation.

CONCLUSIONS AND RECOMMENDATIONS

Sufficient mtDNA diversity exists in Rocky Mountain sheep for restriction enzyme analyses to be useful as a conservation genetics tool. Base-line data on mtDNA diversity in transplant source-herds can be useful for monitoring loss of genetic variation in reintroduced herds. The preliminary characterization of mtDNA diversity in several major source-herds (Castle Reef, Gibson Reservoir, BLM, Torry Rim, and Tarryall), should be useful in detecting a loss of genetic variation in the many herds founded from these sources.

If females are used for translocations, mtDNA analysis would be useful for detecting the genetic contribution of supplemented individuals into existing gene pools and,

consequently, the success of a translocation. This study revealed that some transplant source-herds (e.g., Tarryall) have distinct haplotypes. This information would be useful for detecting reproduction of transplanted females. Females should be preferred for supplemental transplanting because they are more sedentary and more likely to stay in the transplant area (Wilson and Douglas 1982; Dodd 1983; and deVos et al.1991) and because reproductive success is more predictable in ewes than in rams. Since only a few dominant males may breed most or all females (Geist 1971, Hogg 1984 and 1988), transplanting males may be an all-or-nothing situation in terms of genetic contribution to future generations.

MtDNA diversity estimates, in conjunction with nuclear gene diversity estimates, can be useful for identifying populations with high (or low) levels of genetic variation. Because mtDNA is essentially one locus and mtDNA variation may not reflect nuclear genes, management decisions should be based on data from many nuclear genes as well as from mtDNA (but see Ashley et al. 1990). Herds with high (or low) genetic variation could be favored (or disfavored) for use as a source-herd for transplanting. Herds with low genetic variation could be classified as candidates for receiving supplemental transplants of bighorn sheep, especially if these herds have recently become isolated or suffered population bottlenecks.

Although mtDNA variation may not always reflect nuclear gene variation, conservation genetics studies would benefit by including mtDNA analyses because: mtDNA analyses are generally easier to conduct than nuclear DNA studies, mtDNA variation is often more sensitive to population bottlenecks, and because mtDNA analysis provides a unique and independent estimate of genetic variability. Studies of mtDNA diversity in conjunction with studies of nuclear gene diversity are recommended to thoroughly monitor for loss of genetic variation in reintroduced herds.

MtDNA evidence of population substructuring, the evidence for nuclear-encoded allozyme differentiation on a microgeographic scale (Hogg et al. unpublished data) and the evidence of local adaptation in bighorn sheep (Wehausen 1991) suggest a need to study the extent of population differentiation for nuclear genes. Protein electrophoresis studies would not be sufficient because some herds have only one detectable polymorphic protein-coding gene (Hogg pers. comm.). Ideally, analyses of quantitative characters (e.g., lambing date, disease resistance, morphology, etc.) should be included in studies of population differentiation, especially if transplants over long-distances are being considered.

Studies of DNA sequences that evolve at different rates (i.e., mtDNA, hypervariable minisatellite DNA, moderately variable microsatellites, single-copy nuclear sequences)

could help determine how long (i.e., hundreds verses thousands of years) populations have been substructured. Information on the duration of population substructuring would help determine the probability of inbreeding depression and local adaptation in mountain sheep herds.

The mtDNA restriction fragment patterns reported here can be useful for forensic investigations. Species-specific mtDNA restriction fragment patterns have been used in two forensic cases involving confiscated meat from two suspected illegal big game animal kills in Montana (Cronin 1991b). MtDNA restriction-enzyme analysis revealed that one illegal kill was a bighorn sheep and that the other kill was an elk. MtDNA analysis may even be useful for identifying the geographic region from which bighorn sheep were taken because this study showed that different regions may have unique mtDNA haplotypes.

Many mtDNA phylogeography studies have included only a few individuals per sampling location. Because this study and other studies of mobile species have discovered phylogenetically divergent haplotypes within local populations, studies of mtDNA phylogeography in mobile (or potentially mobile) species should include large intrapopulation sample sizes.

Finally, most mtDNA studies measure mtDNA variation as sequence divergence (P-values) and haplotype richness; few

studies report haplotype diversity estimates. Haplotype diversity (h) is less dependent on sample size and is, therefore, a better measure for comparing data from different populations and studies (Chakraborty and Danker-Hopfe 1991).

TABLE 1. MtDNA restriction fragment patterns monomorphic in *O. c. canadensis*. All restriction enzymes are 6-base cutters. One Dall sheep was analyzed for use in phylogeny reconstruction (i.e., for rooting the *O. c. canadensis* phylogeny).

Enzyme	Fragment lengths (kilobases) *O. c. canadensis*	*O. dalli*	Enzyme	Fragment lengths *O. c. canadensis*	*O. dalli*
Ava I	N/C	N/A	Hind III	N/C	N/A
			Mlu I	N/C	N/A
Bcl I	10.0	N/C	Kpn I	N/C	N/A
	5.2				
	0.8		Nhe I	---	11.15
				11.1	---
				4.9	4.9
Bgl I	9.3	9.3	Pvu I	10.5	10.5
	6.85	6.85		5.5	5.5
Bgl II	6.6	6.6	Stu I	4.6	- - -
	4.15	4.15		3.75	3.75
	4.1	4.1		- - -	3.3
	1.76	1.76		3.25	3.25
				2.7	2.7
Dra I	4.1	4.1		---	1.3
	3.7	3.7		0.8	0.8
	2.15	2.15		0.66*	
	1.85	1.85		0.63*	
	1.50	1.50			
	1.3	1.3	Xba I	10.0	10.0
	0.75*	0.75*		3.15	3.15
				1.85	1.85
				1.17	1.17
Eco RI	- - -	+/-13.0			
	9.0	- - -			
	3.9	- - -	Pst I	N/C	N/A
	2.8	2.8			
		0.3*			
			Eco RV	N/C	N/A
Hind II	- - -	8.0			
	5.2	- - -			
	3.2	3.2			
	2.8	- - -			
	1.675	1.675			
	1.2	1.2			
	- - -	0.80			
	0.75*	- - -			
		0.65*			

* = fragments too small for consistent resolution, and therefore not used in data analyses.
N/C = (non-cutting) enzymes that cut at 0 or 1 restriction site. Once-cut fragment patterns are difficult to distinguish from non-cut patterns.
N/A = not assayed.

TABLE 2. MtDNA restriction fragment patterns polymorphic in *O. c. canadensis*. Fragment sizes are in kilobases. All *O. dalli* fragment patterns were either the same as pattern "A" in *O. canadensis*, or different and labeled pattern "G".

Enzyme	Fragment pattern						
	O. c. canadensis						*O. dalli*
	A	B	C	D	E	F	G
Bam HI	- - - -	- - - -					9.5
	5.8	5.8					- - - -
	4.2	4.2					4.2
	3.74	3.74					- - - -
	1.37	1.37					1.37
	- - - -	1.25					- - - -
	1.15	- - - -					1.15
Hae III4	1.85	1.85					1.85
	1.38	1.38					- - - -
	1.21	- - - -					1.21
	1.15	1.15					1.15
	0.99	0.99					0.99
	- - - -	0.91					- - - -
	- - - -	- - - -					0.82
	0.80	0.80					0.80
	0.69	0.69					0.69
	- - - -	- - - -					0.56
	0.55	0.55					0.55
	0.495	0.495					0.495
Hinf I4	1.83	1.83	1.83	1.83	1.83	1.83	- - - -
	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	1.73
	- - - -	----	----	----	----	- - - -	1.70
	- - - -	- - - -	----	1.42	- - - -	- - - -	- - - -
	1.38	----	1.38	- - - -	1.38	1.38	1.38
	- - - -	- - - -	- - - -	----	----	1.13	- - - -
	- - - -	1.125	----	- - - -	- - - -	- - - -	- - - -
	1.075	1.075	1.075	1.075	1.075	1.075	- - - -
	0.90	0.90	0.90	0.90	0.90	0.90	- - - -
	- - - -	- - - -	----	- - - -	0.88	- - - -	- - - -
	0.75	0.75	0.75	0.75	0.75	0.75	0.75
	- - - -	- - - -	0.70	- - - -	- - - -	- - - -	0.70
	0.65	0.65	0.65	0.65	0.65	0.65	- - - -
	- - - -	- - - -	----	- - - -	0.61	- - - -	- - - -
	0.58	0.58	0.58	0.58	- - - -	- - - -	0.58
	0.55	0.55	0.55	0.55	- - - -	- - - -	0.55
	0.51*	0.51*					
	0.495*						
	0.047*						
	0.40*						
	0.35*						
	0.29*						

TABLE 2. Continued

Enzyme	Fragment pattern						
	O. c. canadensis						*O. dalli*
	A	B	C	D	E	F	A, B or G
Hpa I	8.5	8.5					8.5
	3.25	3.25					3.25
	1.65	1.65					1.65
	1.50	1.50					1.50
	- - - -	1.20					- - - -
	0.80	----					0.80
Nci I	10.4	10.4					10.4
	- - -	2.9					- - - -
	2.5	2.5					2.5
Sca I	7.1	7.1					7.1
	3.65	3.65					3.65
	2.8	2.8					2.8
	- - - -	2.275					2.275
	2.2	----					----
Sty I5	3.52	3.52					3.52
	2.97	2.97					2.97
	- - - -	2.75					- - - -
	1.91	1.91					1.91
	1.75	1.75					1.75
	1.53	1.53					- - - -
	1.3	1.3					1.3
	0.62	0.62					0.62
	- - - -	----					0.60
	0.59	0.59					0.59
		0.49					
		0.48*					
		0.3*					

* = fragments too small for consistent resolution and not used in data analyses.
4 = base cutter
5 = base cutter (all other enzymes = 6-base cutters)

Table 3. Eleven *O. c. canadensis* and one *O. dalli* mtDNA genotypes (composite haplotypes) used in phylogenetic analyses. Each haplotype represents a unique combination of the different restriction fragment patterns discovered. "A" was arbitrarily assigned to the most common pattern. Letters are the same as in Tables 1 and 2. Dashes ("-") represent the "A" pattern. A total of the 63 individuals were analyzed with enough diagnostic enzymes to determine composite haplotypes (see "Total # identified"). Frequency heterogeneity analyses inbolbed only five haplotypes (AAA, ABA. AAB, BAA, BAB) which were identified by the first three enzymes listed (i.e., Hae III, Sty I and Bam HI).

Haplo-	Restriction Enzyme & Fragment Pattern												Total #
type #	HaeIII	StyI	Bam HI	HpaI	Hinf I	Nci I	Sca I	EcoRI	HindII	NheI	Stu I	BclI	identified
1	A	A	A	A	A	A	A	A	A	A	A	A	25
2	-	B	-	-	-	-	-	-	-	-	-	-	7
3	-	-	-	-	-	B	-	-	-	-	-	-	1
4	-	-	-	-	B	-	-	-	-	-	-	-	3
5	-	-	-	-	F	-	-	-	-	-	-	-	1
6	-	-	B	-	D	-	-	-	-	-	-	-	2
7	B	-	-	B	-	-	-	-	-	-	-	-	3
8	B	-	-	B	C	-	-	-	-	-	-	-	17
9	B	-	B	B	E	-	-	-	-	-	-	-	1
10	B	-	B	B	C	-	B	-	-	-	-	-	2
11	B	-	B	B	F	-	B	-	-	-	-	-	1
Dall	G	G	G	A	G	A	G	G	G	G	G	G	

TABLE 4. Geographic distribution and number of individuals used for assessing sequence divergence (P-values), phylogeography and haplotype frequency differentiation. All herds are indigenous except Rampart. Frequency differentiation was assessed among subpopulations and among six regional groups of subpopulations. Superscript numbers (1-6) designate the regional group in which each subpopulation was grouped.

Name of local subpopulation	Location	Number of individuals used to assess: P-values	phylo-geography	frequency differentiation
1. Cadomin	Alta[1]	5	5	9
2. Ram Mountain	Alta[1]	0	0	19
3. Sheep River	Alta[1]	1	2	15
4. Columbia Lake	BC[1]	3	3	3
5. Primier Ridge	BC[1]	3	3	3
6. Pot Shot Creek	MT[2]	0	2	2
7. Deep Creek	MT[2]	1	1	3
8. Castle Reef	MT[2]	3	6	41
9. Gibson Reservoir	MT[2]	1	3	36
10. Ford Creek	MT[2]	3	7	14
11. Salmon River	ID-MT[3]	5	5	7
12. W. Fk. Bitter-root River	ID-MT[3]	1	1	1
13. Spanish Peaks	MT[4]	3	3	7
14. Tom Minor	MT[4]	1	1	1
15. Gardiner	MT[4]	0	1	1
16. N. Fk. Shoshone River	WY[5]	0	1	1
17. S. Fk. Shoshone River	WY[5]	1	3	16
18. BLM	WY[5]	0	1	36
19. Torry Rim	WY[5]	1	4	25
20. Tarryall	CO[6]	6	10	22
21. Pikes Peak	CO[6]	1	1	1
22. Cottonwood Creek	CO[6]	0	0	25
23. Rampart (transplanted)	C O*	0	0	19
	TOTALS* =	39	63	292

* = Because the Rampart herd is not indigenous, it was not included in the totals or used in assessing P-values, phylogeography and frequency heterogeneity.

TABLE 5. MtDNA sequence divergence and haplotype richness within North American ungulate species. Maximum percent sequence divergence (divergence between the two most divergent haplotypes) is used here because the average sequence divergence is seldom published. Estimates of sequence divergence and haplotype richness are affected by geographic range of sampling, number of restriction enzymes used, and sample size.

Species	Geographic range sampled	# of Total animals sampled	# of haplotypes identified	Maximum % Sequence Divergence	Reference
Woodland & barren ground caribou (2 subspecies*)	Alaska - Main > 8 locals	81	13	1.4	Cronin 1991
Moose (4 subspecies^^)	Alaska - New Hampshire > 8 locals	32	1	0.0	Cronin 1991
Elk (5 subspecies^)	British Columbia -Colorado-Asia	28	3	0.1	Cronin 1991
White-tail deer *(O. virginiana)*	N. Minnesota 5 locals	309	11	< 1.0	Cronin et al. 1991a
White-tail deer *(O. virginiana)*	Main-S. Carolina -Washington	46	4	1.2	Cronin et al. 1988
Mule deer *(O.h. hemoinus)*	Montana 2 locals	226	3	<1.0	Cronin et al. 1991a
Dall sheep *(Ovis dalli)*	Brooks Range & Alaska Range, AK	4	2	1.2	Ramey 1990
Desert sheep (1 subspecies@)	California - Utah	21	4	0.2	Ramey 1990
Rocky Mtn. sheep	Alberta-Colorado 22 locals	292	11	0.78	This study

* = *Rangifer tarandus caribou* and *R.t. granti*
^^ = *Alces alces andersoni, A.a.shirasi, A.a.gigas, & A.a.americana.*
^ = *Cervus elaphus nelsoni, C.e.roosevelti, C.e. manitobensis, C.e.nannodes, C.e.canadensis.*
@ = Ramey (in prep.) revised desert sheep taxonomy: all are *Ovis canadensis nelsoni*

Table 6. Haplotype diversity in 15 indigenous and one reintroduced Rocky Mountain sheep populations. Diversity was calculated using the 3-enzyme haplotypes from Figure 4 and Birky's (1983) gene diversity equation. Gene diversity is equivalent to heterozygosity and the range of possible values is 0-1.

Population	Sample size	Haplotype diversity	Number of haplotypes
Cadomin	9	0.00	1
Ram Mtn.	19	0.11	2
Sheep River	15	0.13	2
British Columb.	6	0.00	1
Deep Creek	8	0.67	3
Gibson	37	0.55	3
Castle Reef	41	0.65	3
Ford Creek	15	0.26	3
Salmon River	7	0.00	1
Spanish Peaks	9	0.22	2
Shoshone	17	0.00	1
Torry Rim	36	0.00	1
BLM	25	0.11	2
Cottonwood	26	0.00	1
Tarryall	22	0.61	3
Rampart (transplanted from Tarryall)	18	0.11	2

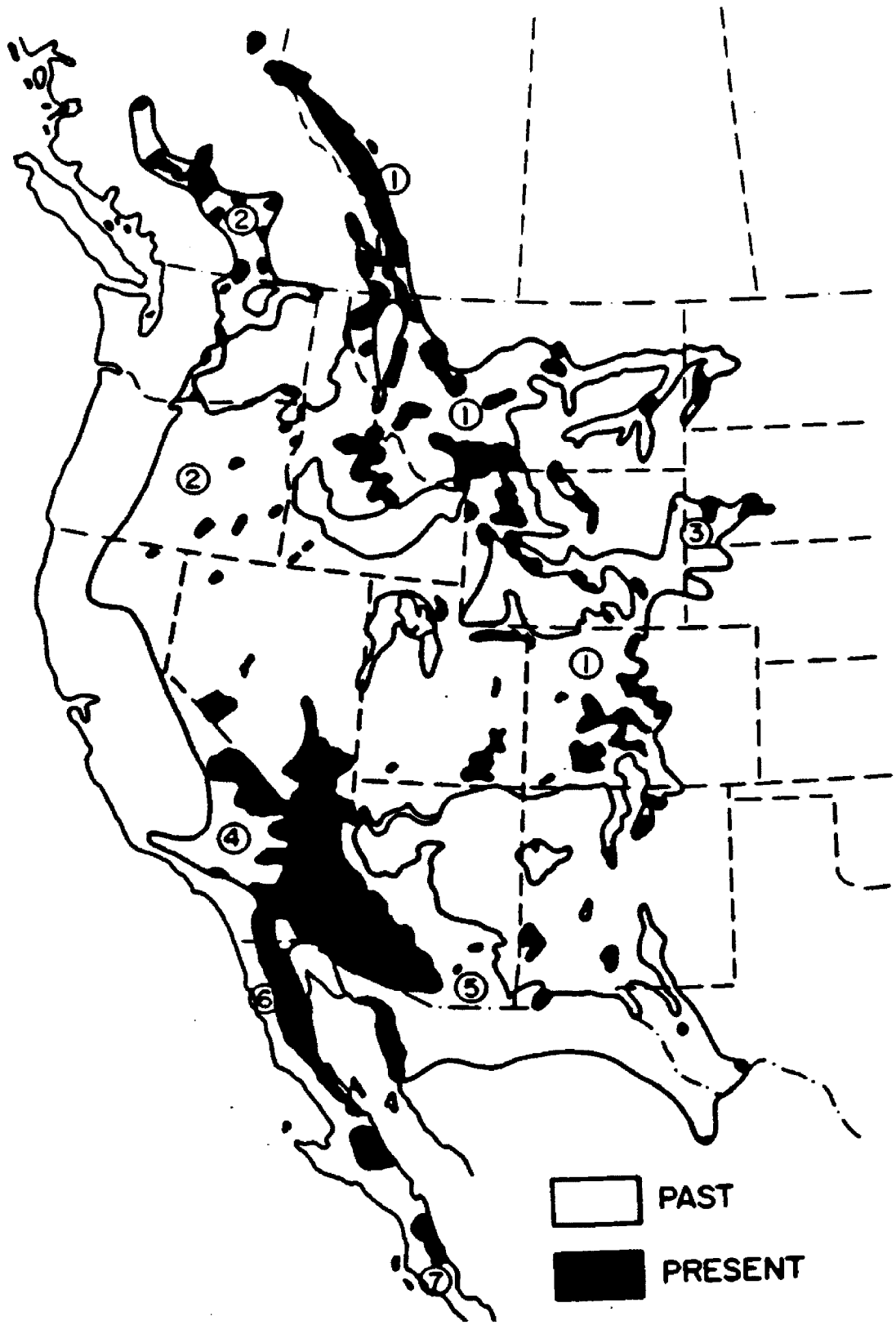


Figure 1. Past (before 1800) and present (1970s) distributions of bighorn sheep (from Wishart 1978; after Cowan 1940, Buechner 1960, and Trefethen 1975): 1. *Ovis canadensis canadensis*; 2. *O. c. californiana*; 3. *O. c. audoboni* (extinct); 4. *O.c. nelsoni*; 5. *O. c. mexicana*; 6. *O. c. cremnobates*; and 7. *O. c. weemsi*. See Ramey 1991 and Ramey in prep. for a revised classification of desert bighorn sheep (i.e., 4-7).

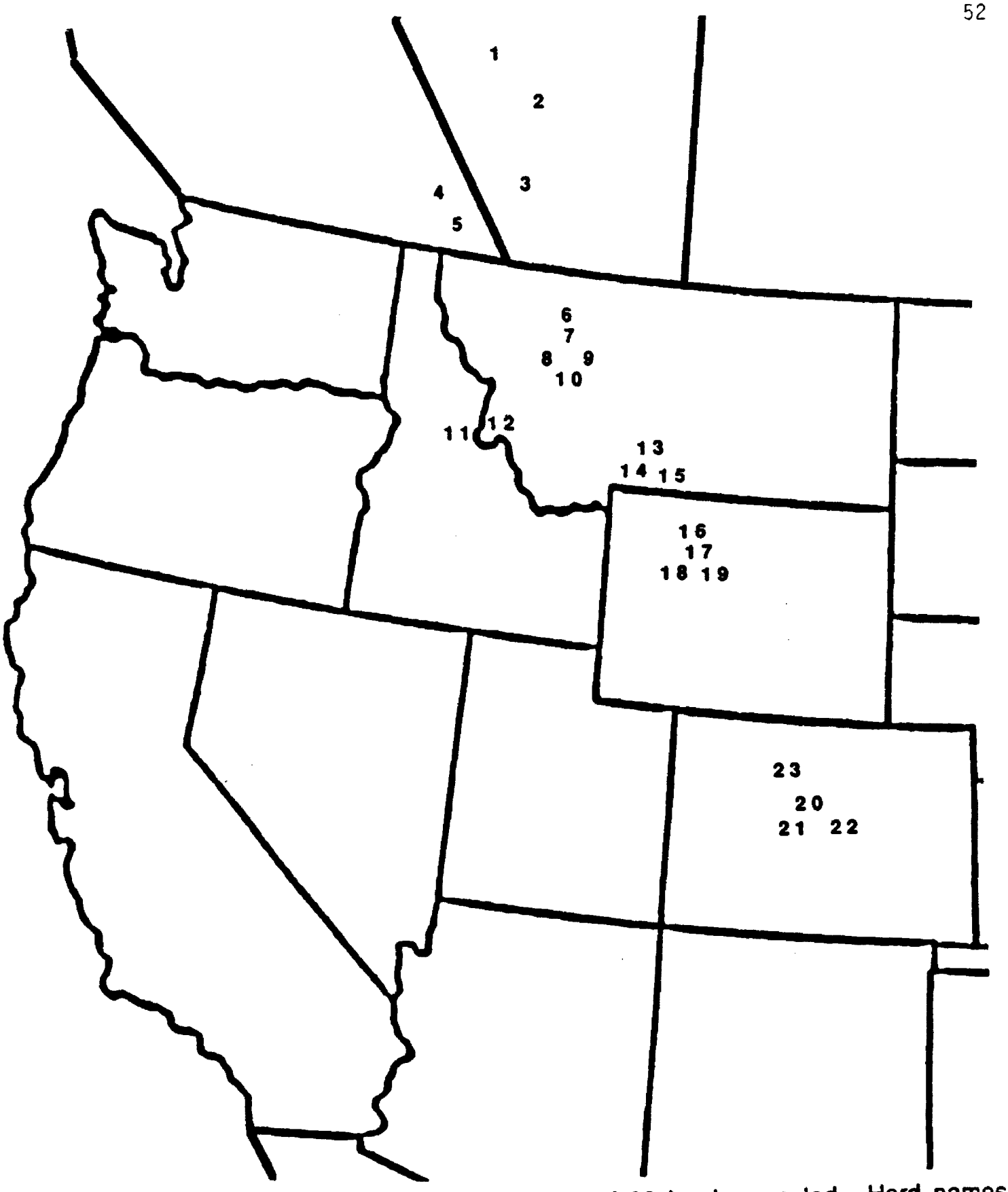


Figure 2. Approximate locations of 23 herds sampled. Herd names are listed in Table 4.

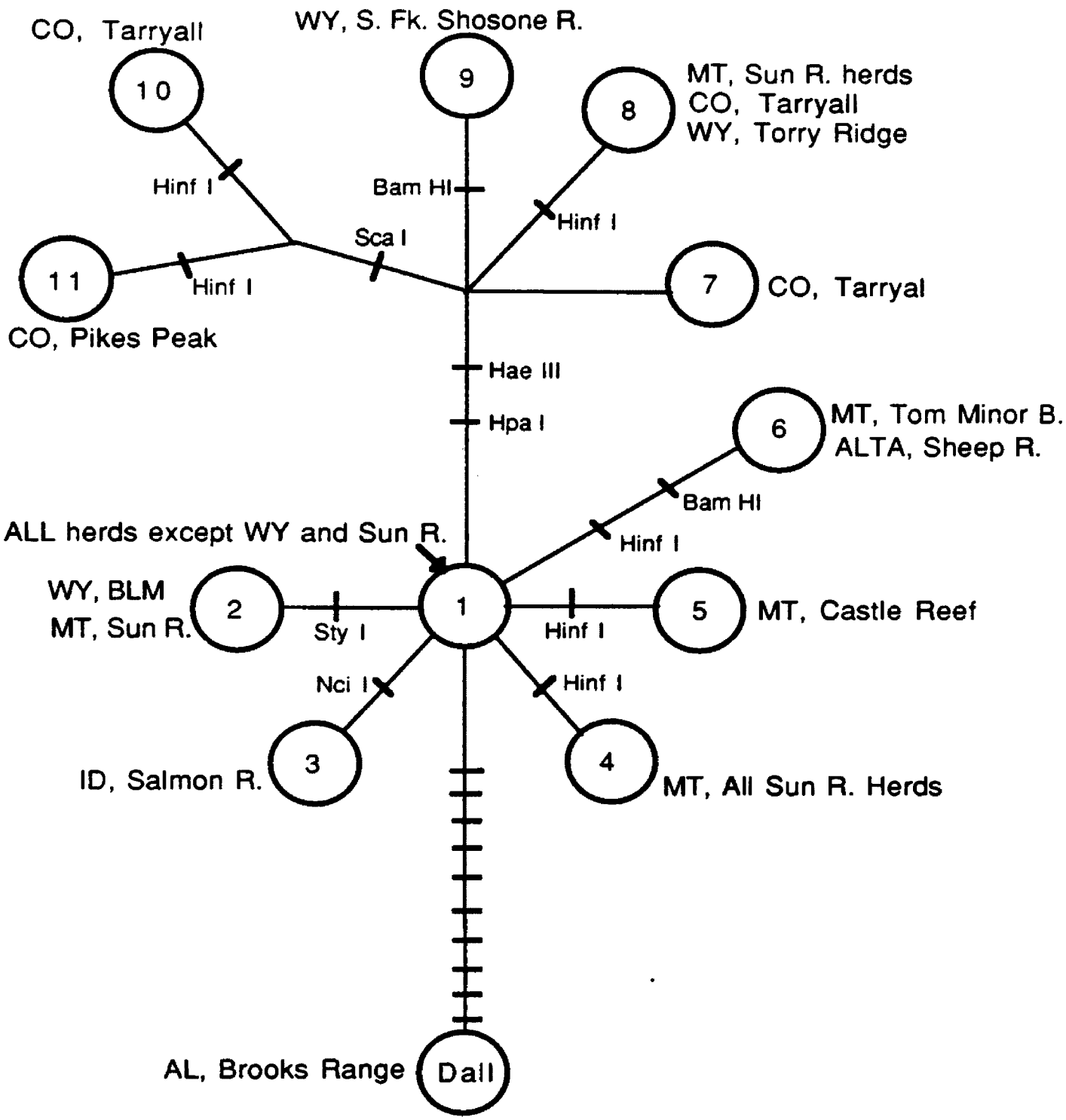


Figure 3. Phylogenetic network of eleven Rocky Mountain bighorn sheep haplotypes generated from restriction fragment pattern data and the strict consensus tree option of PAUP 3.0 (Swofford 1992). A Dall sheep was used as an outgroup to root the network. Slashes on branches represent fragment pattern transformations. The geographic distribution of each haplotype (including states, provinces and herd names) is written adjacent to each haplotype. State and provincial abreviations are listed in Table 4. Note that haplotypes #1 and #8 are geographically widespread.

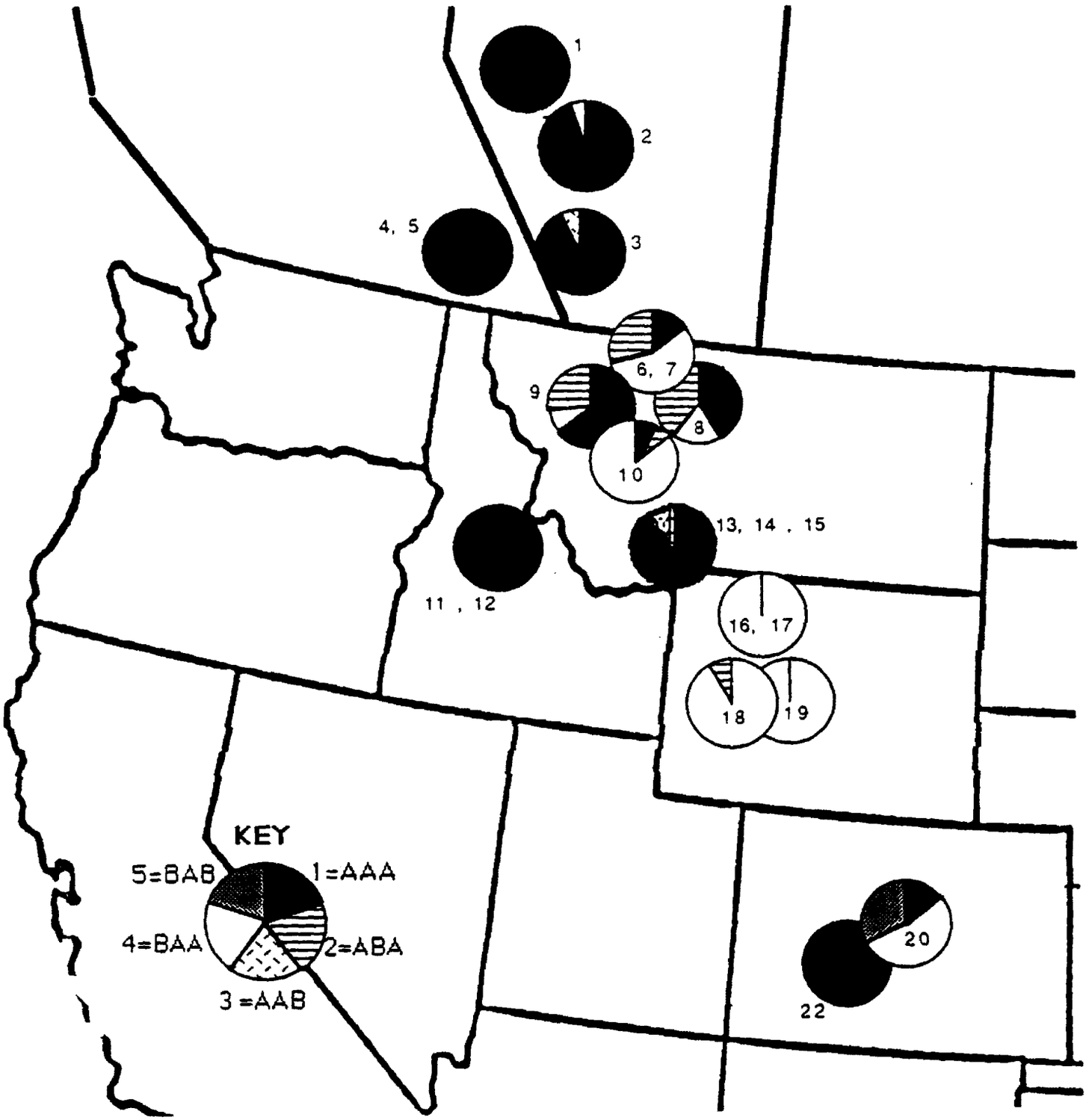
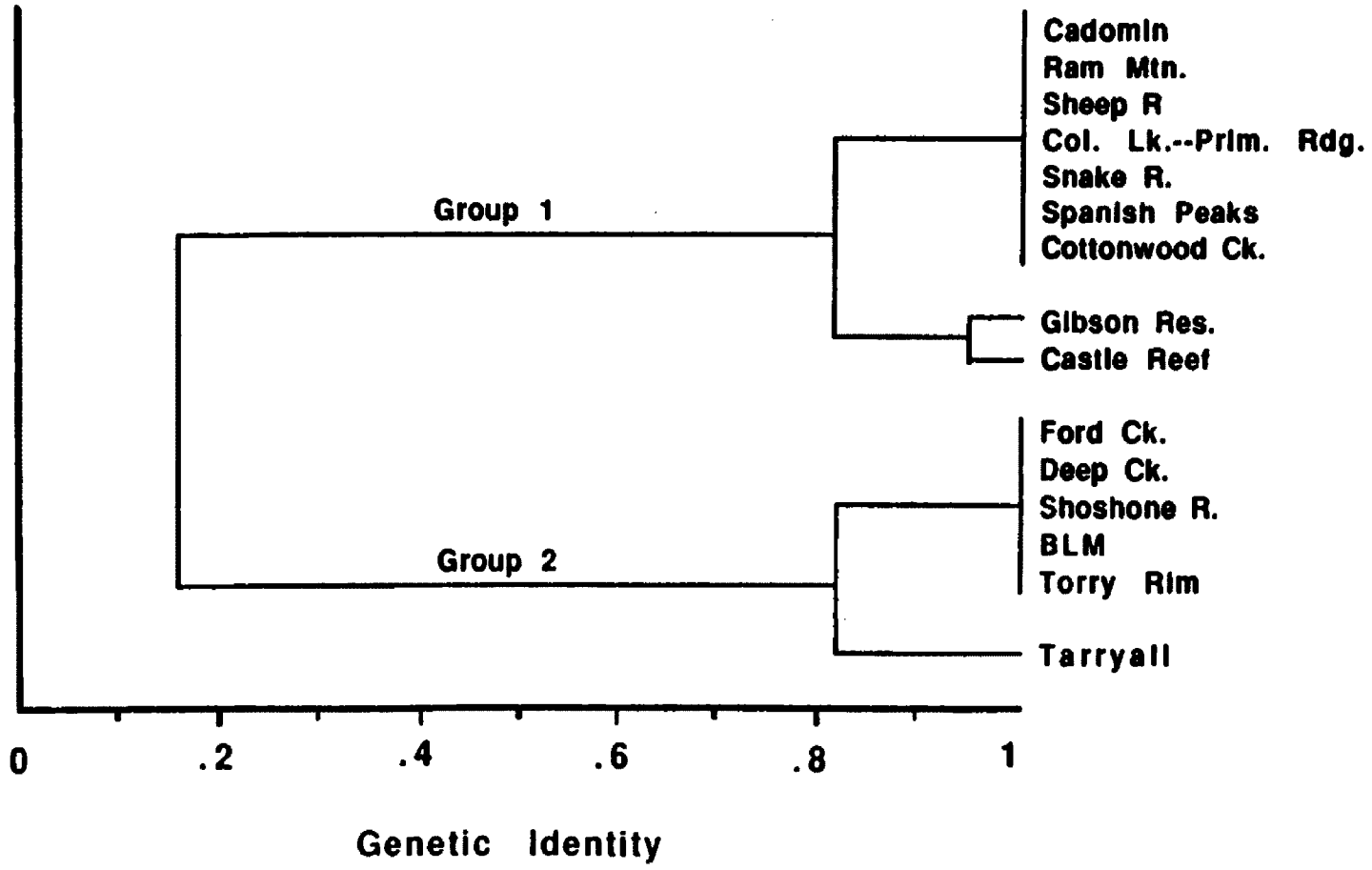


Figure 4. Frequencies of five haplotypes in each of 15 indigenous populations. Numbers refer to herds listed in Table 4. Occurrence of more than one number indicates that subpopulations were pooled. The letters in the key above indicate the fragment paterns produced by restriction enzymes Hae III, Sty I, and Bam HI (Table 3).

Fig. 5. Genetic Relationship (UPGMA cluster) of Rocky Mountain sheep herds based on Nei's unbiased identity of mtDNA frequencies.



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