

TESTING FOR WOLF-COYOTE HYBRIDIZATION IN THE ROCKY MOUNTAINS USING MITOCHONDRIAL DNA

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Abstract: Hybridization between gray wolves (*Canis lupus*) and coyotes (*Canis latrans*) has been documented in the Great Lakes region of the United States and Canada but has not been extensively studied in the Rocky Mountain region. We used mitochondrial DNA (mtDNA) to evaluate potential gray wolf-coyote hybridization in wolf populations in the western United States, Alberta, and British Columbia, including wolves reintroduced into Yellowstone National Park (YNP) and central Idaho. A restriction site and a length difference in the control region (D-loop) of mtDNA was used to differentiate wolf and coyote haplotypes. All 90 wolves tested had wolf haplotypes. We concluded that the wolf populations in the Rocky Mountain region have not hybridized with coyotes as they have in the Great Lakes region. This method could be used to test other wolf populations for wolf-coyote hybridization and monitor the translocated YNP and Idaho populations in the future.

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Evidence of hybridization in the wild among species of Canidae has been found via molecular genetics (Lehman et al. 1991, Gottelli et al. 1994, Roy et al. 1994). Hybridization between gray wolves and coyotes has occurred in the Great Lakes region of the United States and Canada (Lehman et al. 1991, Wayne et al. 1991, 1992, Roy et al. 1994). In this area of introgressive hybridization, coyote haplotype frequency in wolf populations was >50%, yet wolf haplotypes were not found in coyote populations (Lehman et al. 1991). Multiple coyote mtDNA haplotypes found in wolves indicate at least 6 hybridization events occurred in the wild (Lehman et al. 1991). The unidirectionality of wolf-coyote matings (a male wolf mating with a female coyote), however, is not well understood, yet viable hybrids apparently backcross to wolves but not coyotes (Lehman et al. 1991, Roy et al. 1994, Wayne 1996).

Coyote mtDNA introgression into wolf populations has been documented in the Great Lakes region, but it has not been extensively tested in the Rocky Mountain region. An initial study included 6 wolves from Montana, 4 from Alberta, and 2 from Manitoba, none of which had coyote haplotypes (Lehman et al. 1991). Differentiating wolves and coyotes genetically and detecting coyote genes in wolf populations

is valuable for management. For example, the reestablishment of pure gray wolf populations in the Rocky Mountain region is important for the eventual delisting of this endangered species, but introgressive hybridization between gray wolves and coyotes would threaten the recovery of locally endangered gray wolf populations and complicate management.

Wolf recovery in Montana, southwest Alberta, and southeast British Columbia has been slow yet dramatic following repeated extirpations in the 1930s and 1950s (Boyd et al. 1995). Wolves began to naturally recolonize the areas of Banff National Park, Alberta (BNP), and Glacier National Park, Montana (GNP), via dispersal from populations further north in Canada during the 1970s and 1980s. The current population estimate in western Montana is about 80-100 wolves with 8-10 breeding pairs (Bangs and Fritts 1996). The chances for survival of the gray wolf in the Rocky Mountains are favorable due to the Endangered Species Act listing of the gray wolf as endangered in the lower 48 states in 1973 (U.S. Fish and Wildlife Service 1987), changing public attitudes (Mech 1995), and the recent reintroductions of wolves into YNP and central Idaho (Fritts et al. 1995, 1997). However, given that coyote introgression has occurred in wolf populations in other areas, the possibility of interbreeding is an ongoing concern.

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Genetic markers vary in their ability to detect species differences. Allozyme studies are unlikely to detect wolf-coyote hybridization because relatively little genetic variation is found within wolves (Kennedy et al. 1991, Wayne et al. 1991), and there is little allozyme differentiation between wolves and coyotes (Ferrell et al. 1978, Wayne and O'Brien 1987). Allozyme analyses also require relatively large amounts of tissue and potential sacrifice of individuals.

In contrast, polymerase chain reaction (PCR; Mullis et al. 1994) techniques require minuscule amounts of blood or tissue and can use numerous polymorphic genetic markers. Analysis of mtDNA is useful for investigating genetic differences between closely related species (Kocher et al. 1989, Avise 1994), provided thorough testing of haplotype distributions has been conducted. Due to its maternal inheritance and lack of recombination, mtDNA can be used to detect past hybridization events. The noncoding control region (D-loop) of mtDNA is especially valuable for population genetic studies in mammals (Randi et al. 1994, Slade et al. 1994, Taberlet et al. 1995). In mammals, this region mutates faster than the rest of the mtDNA molecule or single-copy nuclear DNA (Avise 1994) and is therefore often more polymorphic and informative for detecting species differences.

Use of mtDNA has previously detected introgressive hybridization in wolves in the Great Lakes region (Lehman et al. 1991). However, to determine whether introgressive hybridization detected with mtDNA was an ancient event or is ongoing, biparentally inherited nuclear markers such as microsatellite loci (hypervariable, tandem repeats) are needed. A previous microsatellite study on wolves and coyotes in the Great Lakes region demonstrated nuclear gene introgression, with allele frequencies affected in wolf but not coyote populations (Roy et al. 1994). This finding further supports the use of mtDNA as an adequate marker for detecting introgressive hybridization between these 2 species, given the behavioral pattern of hybridization. A recent microsatellite study on wolves from the Rocky Mountain region (Forbes and Boyd 1996, 1997) provided baseline nuclear data for future monitoring of these wolf populations, should introgression be detected with mtDNA.

We used the mtDNA control region (D-loop) to investigate whether the same pattern of wolf-coyote hybridization found in the Great

Lakes region is evident in Rocky Mountain wolves. We sequenced the control region in wolves and coyotes from the Rocky Mountain region to detect diagnostic genetic differences between wolf and coyote mtDNA. We compared our sequences to wolf and coyote mtDNA sequence data from throughout each species' range to verify the global conservation of species-specific sequences. We then tested for coyote mtDNA introgression in wolves from populations in Montana, Alberta, and British Columbia, as well as wolves recently translocated to YNP and central Idaho.

STUDY AREA AND METHODS

We analyzed mtDNA of 90 wolves (46 from northwestern Montana, 19 from the BNP area, 13 from near Hinton, Alberta, and 12 near Fort St. John, British Columbia) previously studied via DNA microsatellites (Forbes and Boyd 1996, 1997). The Hinton and Fort St. John areas were sources of wolves trapped for translocation to YNP and central Idaho in 1995 and 1996 (Fritts et al. 1997). Our sample represents wolves from 14 of the 18 packs used for translocation. We sampled 30 coyotes from northwestern and central Montana.

We collected blood from live, wild wolves and muscle from wolves and coyotes found dead (Ream et al. 1991, Boyd et al. 1995). We isolated genomic DNA via either genomic DNA or Chelex preparations (Forbes and Boyd 1996). We used PCR to amplify about 400 base pairs (bp) of the control region via universal mtDNA primers L15905 and H16517 (Gottelli et al. 1994). Ten- μ L PCR reactions contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, 2 μ g/mL BSA, 0.2 mM of each dNTP, 4 pmoles of each primer, 0.5 U Taq DNA polymerase (Perkin-Elmer Cetus, Foster City, California, USA) and either 50–100 ng purified genomic DNA or 1 μ L Chelex tissue preparation. The PCR profile for the universal mtDNA primers was 35 cycles at 92°C for 1 min, 45°C for 1 min, and 72°C for 30 sec.

We ran the PCR products from the universal mtDNA primers on a 2.5% agarose gel containing ethidium bromide in TAE buffer (Ausubel et al. 1989). We performed direct automated sequencing (Applied Biosystems, Foster City, California, USA) on PCR products purified from the agarose gel using the GENE CLEAN Kit (BIO 101, La Jolla, California, USA). We aligned wolf and coyote sequences (Genbank

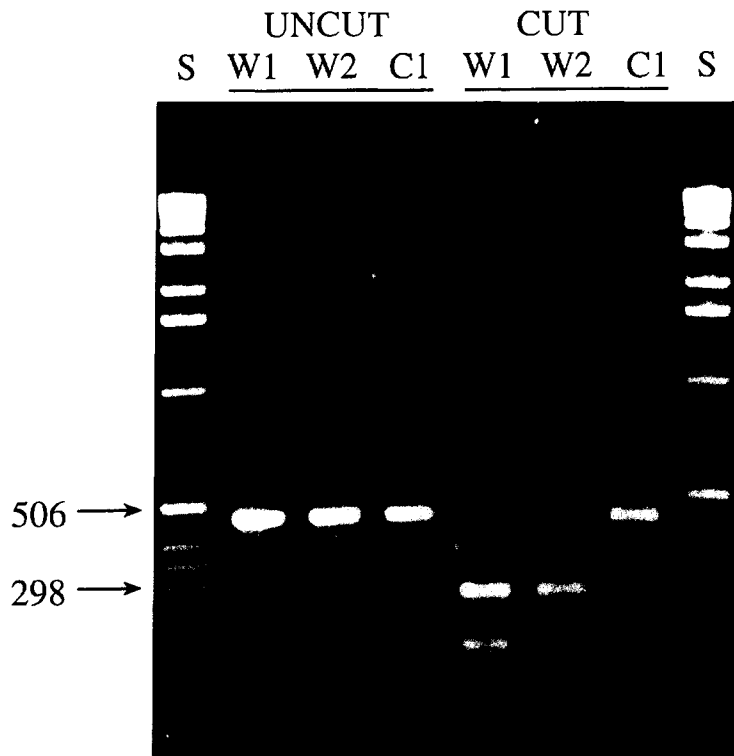


Fig. 2. Agarose gel showing mtDNA fragments from 2 wolves and a coyote amplified via universal mtDNA PCR primers. The first 3 lanes after the standard show the uncut PCR products, and the last 3 lanes are the products cut with *Mva*I from the same individuals. First and last lanes are 1 kb DNA size standards.

due to the rapidly evolving nature of the mtDNA control region.

Aligning sequences, we identified a restriction site present in wolves but lacking in coyotes due to a 2 bp deletion (Fig. 1) To test for wolf or coyote haplotypes, we used restriction enzyme *Mva*I. *Mva*I cuts at cc(a,t)gg and cuts wolf but not coyote mtDNA. We cut universal mtDNA primer products from all 90 wolves into 2 fragments of approximately 280 and 160 bp (Fig. 2). We cut canid-specific primer products from 7 wolves into 2 fragments of 49 and 113 bp. The mtDNA remained uncut in all 30 coyotes tested with both primer sets. Thus, all 90 wolves tested had the restriction site present in wolves that is absent in coyotes.

The canid-specific primers were more efficient than the universal mtDNA primers in amplifying low-quality wolf and coyote samples. The increase in successful amplification may be due to a shorter target DNA template or because these primers more closely match the DNA template sequence. Due to this efficiency, and the ability to distinguish wolf and coyote

uncut PCR products based on size on an acrylamide gel, these primers may be more useful for forensic purposes.

In addition to our samples, we examined control-region sequences of wolf and coyote mtDNA collected by other labs from throughout each species' range. The *Mva*I restriction site was found perfectly conserved in 150 European wolf (*Canis lupus lupus*) mtDNA sequences (E. Randi, Istituto Nazionale per la Fauna Selvatica, Italy, personal communication), and in an additional 162 wolf mtDNA sequences from throughout Europe, Asia, and areas of North America where wolves have had no recent contact with coyotes (C. Vilà, University of California, personal communication). Our wolf sequences perfectly matched wolf haplotype W22 (Vilà et al. 1997), which was found in 5 wolves from Alberta, Montana, and the Northwest Territories, Canada. The *Mva*I restriction site was absent in 13 coyote mtDNA sequences from Mexico, California, Florida, Louisiana, Michigan, Washington, Utah, Manitoba, and 4 un-

known locations (C. Vilà, University of California, personal communication).

DISCUSSION

Genetics

We found that wolf and coyote mtDNAs were distinct and could be differentiated both by a PCR product length difference and by a single mtDNA restriction site. When we used the *Mva*I restriction enzyme, mtDNA from animals with the wolf haplotype gave 2 distinct products, while mtDNA from coyotes remained uncut. The deletion at the restriction site in coyotes assured the test could not be confounded by a single nucleotide difference between species. Therefore, coyotes would not gain this restriction site by a single base-substitution mutation.

While mtDNA is extremely informative for detecting hybridization between species, there are also limitations. The mtDNA is inherited clonally from the mother and does not undergo recombination. Therefore, a mtDNA haplotype could persist in a population for many generations. Consequently, while mtDNA is often able to detect even an ancient hybridization event, a concomitant study of nuclear markers would be needed to detect ongoing hybridization and to assess the degree of introgression at nuclear genes.

Hybridization

Introgressive hybridization between wolves and coyotes has previously been found to be unidirectional, affecting wolves but not coyotes (Lehman et al. 1991, Roy et al. 1994). Coyote mtDNA haplotypes found in wolves suggest initial mating of male wolves with female coyotes, followed by backcrossing of the female progeny to male wolves or to other F_1 hybrids.

While the exact ecological causes of past hybridization are not known, it has occurred in areas where deforestation and a conversion to agriculture has taken place (Lehman et al. 1991, Wayne 1996). Coyotes are opportunistic predators that colonize disturbed habitats where wolf populations have been decimated. In such areas, wolves may be unable to find conspecific mates when population densities are very low, which creates a potential Allee effect for wolves (Allee et al. 1949). In addition to low wolf density, coyotes may greatly outnumber wolves in these areas, and a lone male wolf may more

easily find a female coyote to mate with than another wolf.

Absence of initial matings between female wolves and male coyotes may be due to the evolution of different prey selection, social structure, mating strategy, and dispersal behavior for wolves and coyotes. Coyotes are highly adaptable, medium-sized carnivores that most commonly capture prey smaller than themselves and therefore do not need other pack members to aid prey capture. In contrast, wolves are approximately 3–4 times the mass of coyotes and usually capture prey larger than themselves, which is facilitated by cooperative hunting in relatively large packs (Mech 1970, Moehlman 1989). The reproductive success of a female wolf is greatly increased by other pack members helping with pup-rearing (Harrington et al. 1983, Moehlman 1989). A female wolf may perceive a male coyote as a poor investment and refuse to mate with him.

A common alternative for wolf pack formation involves pack splitting where >1 female in a pack may conceive during a breeding season (Mech and Nelson 1989, Mech 1991). The pregnant, subordinate female may leave the pack and den by herself or she may take a small portion of the pack members with her (Boyd et al. 1993, Boyd and Jimenez 1994). The mate selection process by the pregnant female occurred while she was still a pack member, eliminating the potential for hybridization with coyotes.

Overall, we found there has not been an introduction of coyote mtDNA haplotypes into wolf populations in Alberta, British Columbia, or the recolonized Montana population. It is therefore unlikely that introgressive hybridization between gray wolves and coyotes has occurred in the Rocky Mountain region as it has in the Great Lakes region.

MANAGEMENT IMPLICATIONS

Wolf numbers are increasing in western Montana, and the translocated populations in YNP and Idaho are persisting. These areas contain suitable wolf habitat with a sufficient prey base, appropriate denning sites, and reduced human encounters (U.S. Fish and Wildlife Service 1987). Wolves and coyotes coexist in these regions, and hybridization with coyotes seems unlikely as long as habitat is preserved and wolf numbers are maintained. However, hybridization may yet occur in this region because of

ongoing anthropogenic habitat alterations. We have demonstrated a diagnostic mtDNA test that can detect introgressive hybridization between wolves and coyotes given the behavioral pattern of hybridization between these 2 species. We recommend that mtDNA analyses continue to be conducted on wolves and coyotes throughout the Rocky Mountain region to monitor for possible future introgressive hybridization.

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