COMPARISON OF MITOCHONDRIAL DNA IN WOLVES AND COYOTES IN THE NORTHERN ROCKIES USING THE POLYMERASE CHAIN REACTION

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✦ INTRODUCTION

The purpose of this study was the comparison of mitochondrial DNA (mtDNA) sequences from Northern Montana and Minnesota wolves to the extinct wolves of Yellowstone National Park (YNP). This comparison was intended to provide a genetic basis to identify potential wolf stocks for introduction into YNP. Unfortunately the only extinct YNP specimens available were dried tanned skins from the Smithsonian Museum of Natural History and YNP. The nucleic acids extracted from these skins was so badly degraded that no amplification of mtDNA was possible; so the critical comparisons could not be made.

The control region of the mtDNA is 1100-1200 nucleotides long in vertebrates, contains sequences that control replication and gene activation and in terms of evolutionary analysis has the desired feature of both variable and conserved regions. The variable regions provide the genetic diversity that is the basis of taxonomic and evolutionary studies while the conserved regions provide the ability to synthesize short complementary oligonucleotides to prime the polymerase chain reaction (PCR) in the organisms studies.

✦ MATERIALS AND METHODS

Wolf tissue specimens from the Glacier Park ecosystem were provided by Keith Aune of the MT Fish Wildlife and Parks Department. DNA extracts of some wolves were provided by Steve Fain, of the US Fish and Wildlife Service Forensics laboratory in Ashland, Oregon. Minnesota wolf tissue specimens were kindly provided by the US Fish and Wildlife lab in Minnesota. Norm Bishop and John Varley of YNP kindly obtained skin specimens from wolf skins or mounts in YNP and the Smithsonian Museum of Natural History.

DNA was extracted from tissue samples by the method of Davis et al., (1980). Approximately 1 g of tissue (usually muscle but other tissues work) was chopped finely with a new razor blade in a sterilized plastic petri dish half and then placed in a 2 ml microcentrifuge tube. Extraction buffer of 0.2 M tris-HC1 pH 8.3, 0.1 M EDTA, 650 ul; 10% SDS, 100 ul and Proteinase K 20 ug/ml, 50ul was added to the tissue and then incubated for 1-4 hours at 65 C. The tubes were incubated for an additional 3-24 hours at 35 C. The soft tissue was mashed with a glass pestle which just fits inside the centrifuge tube. Five molar potassium acetate (400 ul) was added and the tubes placed on ice for 30 minutes. After centrifugation at 14,000 g for 8

minutes at least 800 ul of supernatant was decanted into a new 1.5 ml microcentrifuge tube and an equal volume of phenol/chloroform-isoamyl alcohol added. The tubes were inverted to mix the ingredients and then centrifuged at 14,000 g for 12 minutes. Tips were cut off 1 ml plastic pipette tips to provide a wide opening to aid in cleanly removing the aqueous layer from the phenol/chloroform-isoamyl alcohol. The aqueous layer was transferred to a new 1.5 ml centrifuge tube and then extracted with an equal volume of chloroform-isoamyl alcohol. The mixture was centrifuged again at 14,000 g for 5 minutes and the aqueous layer transferred to a new 1.5 ml tube. DNA was then precipitated with an equal volume of isopropyl alcohol at -20 C for 30 minutes. The tubes were then centrifuged at 14,000 g for 25 minutes and the supernatant discarded. The DNA pellet was washed with 200 ul of ice cold 70% alcohol by centrifuging for 4 minutes at 14,000 g and the alcohol was discarded. Pellets were then air dried and dissolved in 200 ul of double distilled sterile water.

Dried skin specimens were extracted in several ways, and first attempts were made to grind small fragments of dried hide in liquid nitrogen with a mortar and pestle. The ground material was then extracted with organic solvents as for tissue samples described above. After consulting with Dr. Steve Fain, at the US Fish and Wildlife Forensic Laboratory, Ashland OR, I spent a week in their laboratory using a freezer mill grinder to crush small fragments of hide and then extracted those extracts with phenol/chloroform as above.

I tried newer and different techniques for forensic specimens as they were published. These techniques included chelex extraction (Walsh et al., 1991) and extraction with glass milk (RPM, Bio 101, Inc. La Jolla CA.).

CHELEX EXTRACTION

Small pieces of hide were ground in an autoclaved, UV sterilized mortar and pestle and then extracted with 350 to 500 ul 5% chelex in 1.5 ml microcentrifuge tube by vortexing briefly and then incubating for -24 hours at 55 C on a rocker table. The chelex mix was then vortexed briefly and heated at 95 C for 5 min in a heat block. The chelex mix was then vortexed again for 30 sec. and centrifuged at 14,000 g for 30 sec. The chelex extract supernatant 5 ul was used directly as a template for PCR (Walsh et al., 1991).

GLASS MILK EXTRACTION (RPM)

Dried skin was ground as above and placed in a 1.5 ml microcentrifuge tube, an equal volume of glass milk spin buffer was added and the mixture shaken on a rocker table at room temp for at least 12 hr. Centrifugation at 14,000 g for two minutes pellets the extract and then the supernatant was discarded. Water (250 ul) was added to elute the DNA, the tube was inverted and vortexed to produce a mixture. Centrifugation at 14,000 g for two minutes pellets the sediment and then the supernatant with the DNA was removed and used as a template for PCR.

Internal Primers used for the PCR reactions in this study were developed by Dr. Steve Fain.

Primers: 5'AAGCCCTTATTGGACTAAGTG 3' SF 50 H
5'TAT(CT)CTTACATAGGACATAT 3' SF 50 L

External Primers sequences for this region were taken from Taberlet and Bouvet (1992;1994).

Primers: 5' ACCTTGGTCTTGTAAGCCA 3' L 15927
5' CTCCACTATCAGCACCCAAG 3' 15995
5' GGAGCGAGAAGAGGTACACGT 3' H 16299
5' CCTGAAGTAAGAACCAGATG 3' H 16498

DNA SEQUENCING

Double stranded PCR products were sequenced with US Biochemicals sequences 2.0 kit with the following modifications kindly provided by G. Ritzal at the University of Alberta. All reactions were carried out in 1.5 ml microcentrifuge tubes. PCR products were separated in a 2% FMC Nuseive agarose gel, visualized with UV light after ethidium bromide staining, the bands cut out, extracted with phenol/chloroform, precipitated and then dissolved in water (Sambrook et al., 1989). For each template, 2.5 ul of the appropriate A, C, G, T, ddNTP termination mix was added to the bottom of four labeled tubes. Twelve ul of purified ds PCR product was mixed with 15 pmole (100 ng) of primer in water, the mixture was heated at 95 C for eight minutes, then flash frozen at -80 C until used in the labeling reaction. A master labeling solution was made up with the following proportions for each template, pure water, 1 ul; 5X labeling mix, 1 ul; 0.1M DTT, 1 ul; Manganese buffer, 0.5 ul; P-32 dATP, 0.5 ul; Sequenase 3 U. The master labeling mix, 4.2 ul was dispensed to the quickly thawed primer template mix and incubated for 2-5 minutes at less than 20 C. Then 4.2 ul of the

labeled primed template mix was dispensed to the side of the A, C, G, T, termination mix tubes, which are warming at 37 C. These tubes were centrifuged for 2 sec. and the contents incubated at 43 C for five minutes. The reaction was stopped by adding 5 ul of formamide dye mix and centrifuging for 5 seconds. The sequencing solutions were heated at 95 C for five minutes before loading on a gel.

✦ RESULTS AND DISCUSSION

First attempts at using PCR to amplify mtDNA employed the primers developed by Kocher et al., (1991) to amplify ribosomal RNA or cytochrome b genes. These primers amplified DNA from the tissue specimens but not from the dried tissue. Realizing that the distances between these primers was too long to expect amplification with degraded DNA, the decision was made to switch to the mtDNA control region where primers spanning shorter regions were being developed. These shorter PCR target regions should have increased the probability of getting a result but unfortunately did not do so (Paabo et al., 1988; Paabo, S, 1989).

DNA extracted from tissues, amplified by PCR and then sequenced produced the sequences presented in Table 1. The four Montana wolves from the same family all had the identical sequence while a Montana wolf sequenced by Dr. Steve Fain differed at four sites. This wolf had the same sequence as three Minnesota wolves and the rest of the Minnesota wolves had mtDNA sequences suggestive of past hybridization with coyotes. Hybridization of wolves and coyotes has been reported in Minnesota, Michigan and Ontario, Canada by Lehman et al. (1991) so the finding of coyote mtDNA sequences in Minnesota wolves is not surprising.

Since wolves from Minnesota do show evidence of past hybridization with coyotes, wolves from Northern Montana and Canada are probably a better source of wolves for transplant into YNP than those from Minnesota. Lehman et al. (1991) did not find evidence of coyote wolf introgression in Alberta but their sample size was only two animals so they may have missed any hybridization in that area. However, the wolf samples were reportedly taken from an area with very little agriculture and few coyotes so the likelihood of hybridization in northwestern Alberta is small (Lehman et al. 1991). The source of the wolves selected for transplants into YNP are from this same area in Alberta so they should show no past hybridization with coyotes.

✦ LITERATURE CITED

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Table 1. *Canis lupus* mtDNA Control region light strand sequences starting near the proline tRNA.

First sequence, MT wolves, (#'s 179649, 8-193A. 8-193B, 179659)
Second squence, Steve Fain's sequence for MT wolf (# 8550)
Third sequence, MN Wolf (8997)
Fourth sequence, MN Wolf (9905)
Fifth sequence, MN Wolf (9362)
Sixth sequence, Coyote

1					60
CACCCCTACT	GTGCTATGTC	AGTATCTCCA	GGTAAACCT	TCTTCCCTCC	CCTATGTACG
**********	**********	**********	**********	**********	**********
**********	**********	**********	**********	**********	**********
**********	******C***	**********	AAAA-----*	*****T*C**	**********
**********	******C***	**********	**A*-----*	*****T*C**	**********
**********	**A***C***	**********	**A**T*---	-****T*-***	**********

61					120
TCGTGCATTA	ATGGTTTGCC	CCATGCATAT	AAGCATGTAC	ATAATATTAT	ATTCTTACAT
**********	**********	**********	**********	**********	**********
**********	**********	**********	**********	**********	**********
**********	****C*****	*-********	**********	**********	**C*******
**********	****C***-*	**********	**********	**********	***T******
**********	****C*****	*-********	**********	**********	**C*******

121					180
AGGACATATT	GACTCAATCC	CACAATTCAT	TGATCTATCA	ACAGTAATCA	AATGCACATC
*********C	A********T	**********	**********	**********	******T***
*********C	A********T	**********	**********	**********	******T***
********C*	*******C*T	T***GC***C	******-***	*****GG*T*	******T***
********C*	A***T**C*T	T***G****C	******-***	******GGA*	******T***
********C*	*******C*T	T***G****C	******-***	***GTTGGT*	******T***

181		
ACTTAGTCCA	ATAAGGGCTT	AATCACCATC
**********	**********	**********
**********	**********	**********
**********	**********	**********
C*****	*********-	-*A*******

Minnesota wolves-(#7962, 8654 and 8707 not shown) are identical to the Montana wolf sequence #8505. Minnesota wolf # 8509 (not shown) is also identical to these sequences with the exception of an additional substitution a C for a T at position 112. Other Minnesota wolves #'s 9905, 9362 plus #8997 and others not shown have base substitutions similar to coyotes with additions and deletions that are commonly found in coyotes but not wolves. Minnesota wolf #9362 has a two base addition between bases 164 and 165 that is not shown in this figure.

Underlined sequences are the priming sites of the internal primers designed by Steve Fain.