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Non-Radioisotopic Microsatellite Genotyping of Timber Wolves (*Canis lupus*) Using Faecal DNA

by

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B.E.S., University of Waterloo, 1994

Thesis
submitted in partial fulfillment of the requirements for
the Degree of Master of Science (Biology)

Acadia University
Fall Convocation 1999

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0-612-45362-6

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Table of Contents

List of Tables v

List of Figures vi

Abstract vii

List of Abbreviations viii

Acknowledgements ix

Introduction 1

Methods 11
- Sample Collection 11
- Extraction Methods 12
  - Phenol/Chloroform Extractions 12
  - Guanidinium Isothiocyanate Extractions 13
  - Potato Flour and Purgene© Extractions 14
- Yield Gels and Dilution of Stocks 15
- PCR Conditions 16
- Gel Conditions and Visualization 16

Results 20
- Locus 377 23
- Locus 250 26
- Locus 109 26
- Locus 123 27
- Locus 200 27
- Locus 173 27
- Locus 213 27
- Locus 204 27
- Product from Faeces 28

Discussion 31

Literature Cited 43

Personal Communication 47

Appendix 48

List of Tables

Table 1. Summary of the loci used during the study. 17

Table 2. Summary of the genotypes identified. 24

List of Figures

Figure 1. Yield Gel for DNA Extracted from Blood 21

Figure 2. Yield Gel for DNA Extracted from Faeces 21

Figure 3. Microsatellite Alleles for Locus 377 25

Figure 4. Microsatellite Alleles for Six Extractions of the same Faeces 25

Figure 5. Microsatellite Alleles for Matched Blood and Faecal Samples 29

Abstract

Microsatellite DNA fingerprinting was used in an attempt to identify unknown scat samples from timber wolves (*Canis lupus*) at the Canadian Centre for Wolf Research (CCWR) with DNA recovered from wolf faeces. Silver staining, a non-radioactive technique which is available to small labs, was used to obtain microsatellite genotypes for many of the wolves. Blood samples from a group of free-ranging wolves in Quebec were also used to validate the technique. Genotypes obtained from DNA extracted from blood and faeces collected from the same individuals always matched indicating accurate amplification of the DNA from faeces. The results are compared to other similar studies and the close overlap of alleles of this study and alleles reported in another study strongly suggest the scoring was accurate. Confirming the CCWR pack pedigree was one of the objectives and although all the individuals were not genotyped the suspected pedigree from observational data at the CCWR matched genotypes that were obtained. While the identification of unknown scats was never attempted due to the difficulty in getting this technique to become routine, the results shown in this study suggest that with future refinement, this technique could be used to identify individual scats.

List of Abbreviations

Bp – Base Pair
BSA – Bovine Serum Albumin
CA – Cytosine-Adenine
CCWR – Canadian Centre for Wolf Research
C-TAB - Cetyltrimethylammonium bromide
DNA -Deoxyribonucleic acid
EDTA – Disodium Ethylene Diamine Tetraacetate
EtBr – Ethidium Bromide
GuSCN – Guanidinium Isothiocyanate
ha – hectare
M - Molar
mt DNA – mitochondrial DNA
μm – micro-metre
μM – micro-Mole
SDS – Sodium Dodecyl Sulfate
SLB – Stool Lysis Buffer
PCR – Polymerase Chain Reaction
T_a – Annealing temperature
TE – Tris EDTA
UV – Ultra-Violet
V - Volt
W - Watt

Acknowledgements

I would like to thank my supervisors Peter McLeod and Marty Snyder for all the friendship, support and encouragement they have given me throughout this study. Also, many thanks go to Will Moger who has always been helpful and supportive. Special thanks goes to Jenny Ryon and all the people involved with the CCWR without them this project would not have happened. Great thanks to Hank Halliday for introducing me to the world of wolves and this project. Sophie Czetwertynski deserves many thanks for collecting wolf blood and faecal samples in Quebec.

To all my friends who have helped along the way, your friendships mean more to me than you know. Most of all, thanks go to Dar for being so patient and supportive during these past years.

INTRODUCTION

The aim of the work presented in this thesis was to develop a technique for identifying the donor animal of collected but unidentified wolf scats. Microsatellite DNA fingerprinting, which will be described in full detail later, was considered the most appropriate technique but has traditionally been restricted to large genetics labs with access to radioisotope labeling. Other visualization techniques exist but had not previously been evaluated for their use in conjunction with microsatellite DNA. This study included an evaluation of DNA visualization techniques available to small labs that might provide the sensitivity required for working with microsatellite repeats.

The need to identify the scats of individual timber wolves (*Canis lupus*) arose from an interest in studying the hormonal profiles of wolves with different social ranks within a pack. The hormonal profiles (the importance of which will be discussed later) were to be measured non-invasively from the faeces of wolves at the Canadian Centre for Wolf Research (CCWR) in Nova Scotia. The social behaviour of the pack has been studied for over 20 years and in an attempt to keep their behaviour close to that of free-ranging animals, all studies involving the animals have been non-invasive and none of the current animals have ever been handled by humans. An ability to identify the donors of faeces, using DNA fingerprints, could provide faecal samples for hormonal assays.

An extension of this approach to free-ranging animals could provide an important source of information. The range traveled by individual wolves could be mapped using scat collected and identified to individuals without the costly and difficult operation of fitting radio collars. More importantly, a quantitative measure of the stress that

individuals are under could be investigated by assaying stress related hormones in the faeces. These measurements could then be correlated to a variety of variables including meteorological events, natural disturbances (e.g. forest fires, floods), human disturbances (e.g. logging operations, trapping, ecotourism activities), prey fluctuations and disease outbreaks.

In this introduction, the background that led to the project of fingerprinting DNA in wolf faeces will be covered. The complex social behaviour of the wolf and the benefits of the measurement of individual hormonal profiles by researchers in an attempt to understand these behaviours will be discussed. Studies that have measured hormones non-invasively and studies that have used faeces as a source material for DNA will be summarized and the exciting possibility of combining the two will be presented.

While the behavioral ecology of the timber wolf (*Canis lupus*) has been extensively studied, few researchers have attempted to explore the individual differences in the behaviour of wolves, much less the relations between variations in hormonal profiles and these behavioural differences. Techniques from other scientific disciplines (e.g. endocrinology and molecular biology) make it possible to obtain critical information that was previously inaccessible to the behavioral ecologist. Measuring hormonal differences may help explain many of the complex behavioural interactions observed in this highly social canid species (Mech 1970).

In social mammals other than humans, understanding the effects of social and environmental stress, in natural contexts, is only possible with measurements of stress related hormones. In stress research, one of the most extensively studied free-ranging

species is the male olive baboon. Researchers have been monitoring the stress-induced suppression of the testicular axis and its relation to the social rank and personality of individual baboons (Saplosky 1991). Anesthetizing an animal with a dart in order to draw blood imposes a stress on the animal. Interestingly, animals of different rank were found to respond differently to the stress. The typical response was a rapid decline in circulating testosterone. However, in the same stressful situation an increase in circulating testosterone was seen in high ranking males, possibly giving those individuals a muscular advantage after a series of aggressive interactions. Sapolsky also found that the stability of the dominance hierarchy plays as important a role in an individual's level of stress as does its actual rank. In periods of social upheaval dominant males did not exhibit the same increase in circulating testosterone as during more stable periods. As well, the personality traits of subordinate males were correlated with testosterone measures and how frequently they were able to establish bonds with females in estrus. Maintaining dominant status can determine important factors such as reproductive potential and access to food so an animal's response to stress can reasonably be assumed to have important consequences.

The relations among social behaviour, stress, reproduction, and hormone secretion in the wolf remain relatively unexplored. By living in large groups, wolves are able to hunt large prey. However, it also creates significant social tension among individuals over mating opportunities and food resources (Mech 1970). This tension may be part of the catalyst for the establishment of dominance hierarchies within each pack.

As with many social carnivores, usually only the dominant female of the wolf pack produces offspring each year (Harrington *et al.* 1982). The absence of reproduction in other females remains to be explained. In communally breeding species it has been hypothesized that reproductive suppression of some females occurs when ecological and demographic conditions would result in restricted breeding opportunities of young, inferior individuals (Elmen and Oring 1977, Creel and Waser 1994). In addition to ecological conditions, Creel and Creel (1991) have shown that reproductive suppression in communally breeding carnivores occurs when reproduction is energetically costly. The costs may reach a point where reproductive individuals require help from other members of the group to reproduce successfully.

The investigation of the mechanisms behind the reproductive suppression of subordinate females in wolf packs and an exploration of possible differences in testosterone levels of males were the catalyst for this research. A better understanding of the mating system and the physiological correlates of stress to reproduction and social rank within the pack would help when interpreting individual and pack behaviour.

Although it is often difficult to assign a position to middle ranking individuals within a pack, the dominant (alpha) and low ranking wolves are easily identified (Fentress *et al.* 1987). There are several ways in which dominant wolves may influence the reproduction of the other members of the pack. Aggression, which peaks in frequency and intensity during the breeding season, is most often directed at low ranking individuals (McLeod *et al.* 1996). Aggression from alpha animals may prevent subordinate females from entering estrus through stress induced suppression of

luteinizing hormone in the period before breeding. Subordinate animals may also be prevented from mating through aggression, including outright attack by dominant individuals when subordinates attempt to copulate (Derix and Van Hooff 1995). The dominant animals might also cause stress-induced abortion during pregnancy, or commit infanticide once young are born (McLeod 1990). Measuring the reproductive hormonal cycles in both the dominant and subordinate females in as natural a setting as possible would help to evaluate the above possibilities.

Measuring hormonal differences among wolves requires a source of material from the animals themselves. In many field and captive situations invasive sampling (such as obtaining blood) can be difficult or even impossible. The necessity for continuous or repeated sampling exacerbates the problems. Endocrinological techniques are available for measuring hormonal changes from materials collected non-invasively such as urine, saliva and faeces (Creel *et al.* 1992, Lasley and Kirkpatrick 1991, Wasser *et al.* 1988). Risler *et al.* (1987) found that estradiol-17ß extracted from the faeces of pigtailed macaques corresponded with serum levels in the same animals and provided a non-invasive means of monitoring estrous cycles. Similar success in monitoring the reproductive status of females has been demonstrated in many other mammals including cattle and muskoxen (Desaulniers *et al.* 1989), maned wolves (Wasser *et al.* 1995), baboons (Wasser 1996) and African wild dogs (Creel *et al.* 1997).

In a recent study involving the timber wolves at the CCRW in Nova Scotia, urine samples from the pack of captive yet unsocialized wolves were collected from urine contaminated snow (McLeod *et al.* 1996). Hormonal analysis of the ratio of the stress

related hormone cortisol to creatinine (to correct for dilution by the snow) showed some differences between subordinate and dominant animals. The lowest ranking female and the second ranking male, whose rank in the pack was the most unstable, had the highest ratios indicating they were under the most stress. Unfortunately, collection of urine in this manner is limited to the winter months when suitable snow cover is present.

The initial intent of this study was to expand the above work to include reproductive hormones while using faeces as a source material. This, combined with the urine samples, would have increased the number of samples collected during the winter and allowed for collection throughout the entire year. Also, it would allow for developmental studies of pups prior to their first winter when the first urine samples become available. Unfortunately, even within the confines of the 2.2 ha compound, collecting faecal material from each individual on a regular basis was not possible; the whole compound cannot be observed from any one point and each animal eliminates infrequently. A method for identifying scats became crucial.

The use of molecular techniques to "fingerprint" individual wolves using DNA extracted from faecal matter was considered as a potential solution. If fingerprints could be obtained from known scats of individuals of a group of wolves, other unknown scats collected in the same area could be matched to the donor. Cells are known to be continuously sloughed off the intestinal tract; it was found that in rat faeces collected over a four hour period, approximately nine million viable cells were present (Davidson *et al.* 1995).

DNA recovered from the faeces of free-ranging animals has been used in molecular studies involving a variety of species including ursids, canids and marine mammals. Mitochondrial and nuclear sequences were amplified by polymerase chain reaction (PCR) from DNA extracted from faeces in a study of the genetics of the European brown bear (Kohn *et al.* 1995). In a different application, scats from the endangered kit fox were differentiated from scats of other fox species, coyotes and dogs. Researchers amplified mitochondrial DNA from the scats and then digested the DNA with restriction enzymes and found unique fingerprints for the kit fox (Paxinos *et al.* 1997).

In marine mammal studies, species, sex and individual identity were assigned to pinniped scat samples collected off northeast Scotland (Reed *et al.* 1997). These scat samples were also analyzed for diet composition which allowed the researchers to examine variation in food consumption among different species. As a non-invasive alternative to taking a skin biopsy from the manatee (*Dugong dugon*), mitochondrial work was performed on faecal samples which could be collected without disturbing the animal (Tikel *et al.* 1996).

Prior publications involving wolf genetics have typically focused on population genetics. A variety of genetic techniques have been used by researchers, including allozyme electrophoresis, mtDNA restriction-site analysis, multilocus hypervariable minisatelite DNA analysis (Wayne *et al.* 1991, Randi *et al.* 1993) and microsatellite analysis (Roy *et al.* 1994).

A technique suitable for this study needed to be compatible with the characteristics of DNA extracted from faeces and to be able to create a unique signature for each individual wolf. DNA recovered from faeces would be a) degraded and fragmented; b) contaminated with prey and bacterial DNA; and c) present in very small quantities. In addition technical constraints required that the DNA be visualized non-isotopically.

Of the available techniques, micosatellite analysis seemed the most compatible with the above parameters. Microsatellites are repeats of short nuclear DNA sequences that are highly polymorphic within populations and have mutation rates as high as 1×10^{-3} per generation (Garcia-Moreno *et al.* 1996). A high degree of polymorphism makes them a popular tool for population genetics (Forbes and Boyd 1996). In this study they could potentially target enough allelic variability within a small pack to supply a unique fingerprint using a limited number of loci. Microsatellite polymerase chain reaction (PCR) primers amplify a 100-300 base pair (bp) region of the entire nuclear DNA containing the repeat. PCR is a process by which millions of copies of a region of DNA can be replicated from only a few copies of target DNA (Mullis *et al.* 1986).

In brief, PCR works as follows. In a thermocycler a mixture of target DNA, copies of two synthetic oligonucleotide microsatellite primers, a thermally stable DNA polymerase, vast quantities of single base nucleotides, and buffering agents are all heated in a small vial to 95°C where denaturing of the target DNA takes place. The mix is then cooled to a temperature where the primers anneal to a complimentary sequence on the target DNA which is adjacent to the microsatellite repeat area. The temperature is raised

to 72°C, and in the presence of the DNA polymerase and the correct buffering, polymerization takes place where single base nucleotides complimenatry to the target DNA are attached to synthetically create the double strand DNA. This building of the second strand occurs across the microsatellite repeat region. A second denaturing and reannealing with another primer and the building of the corresponding strand back on the first then creates a fragment of a fixed length which includes the microsatellite repeats. Repeated cycling in the same manner then exponentially creates millions of copies of the fragments that are all the same length.

Microsatellite primers are typically 18-22 bp long which is long enough to eliminate matching to a random sequence in mammalian genomes. When they are used with high annealing temperatures they provide the specificity needed when attempting to amplify canid DNA contaminated by other sources (Innis and Gelfand 1990). As well, fragmented target DNA can be amplified even if only some of the fragments contain the required region. Extractions of DNA from faeces could also contain DNA from prey consumed by the wolves and DNA from bacteria from both within the wolves' gastrointestinal tract and from the ground where the defecations lay before collection. Canid primers originally developed for domestic dogs (Ostrander *et al.* 1993) were available, which eliminated the very time consuming and expensive procedure of developing them. The primers target regions containing between 10 and 24 (CA) repeats and were known to be successful at amplifying DNA from wolf blood (Forbes and Boyd 1996).

Microsatellites are inherited in a Mendelian fashion, where an individual obtains one allele from each of its parents. Using this information it is possible to perform parental analysis (Craighead *et al.* 1995) and work out a pedigree if enough loci are examined. The actual relations among individuals in a pack can have important social consequences, yet in most studies of wolf social behaviour the genealogical relationships among the animals are usually suspected but are rarely confirmed. Multiple matings, communal rearing of offspring or infanticide (McLeod 1990) are all possibilities which exist and are often difficult or impossible to detect, even in a captive setting. With microsatellite alleles used to identify individual scats it should be possible to determine the pedigree of the CCWR pack. This would add vital information for interpreting the complex social behaviour of these canids (Fentress *et al.* 1987).

In summary, the objectives of this work are as follows: first, to evaluate different methods of DNA extraction from faeces for yield and quality; second, to determine the amount of degradation that a faecal sample can undergo before extraction and PCR amplification is no longer possible; third, to explore gel conditions and the use of non-radioactive visualization methods capable of distinguishing allele differences down to 2 base pairs; fourth, to evaluate the effectiveness of this technique at identifying individual scat samples by amplifying captive and free-ranging wolf scat and blood samples; fifth, to compare results found in this work to published studies; sixth, to ascertain the pedigree of the CCWR wolves.

METHODS

Sample Collection

Blood and faecal samples were collected at the Canadian Centre for Wolf Research (CCWR), Nova Scotia, and from a wild population in the Réserve des Laurentides, Quebec.

At the CCWR, excretions from each of the 13 wolves that were observed and therefore linked to individual wolves, were collected, labeled and stored (for periods ranging from 1 day - 2 years) at -20°C until the extractions were performed. Collection sweeps of the entire 2.2 ha compound were conducted where informal transects ranging from 2 to 5 metres apart were followed depending on vegetation cover. If thick grass was present, as in open areas in the summer, transects needed to be closer together than in wooded areas with little ground vegetation or in the winter when faeces were clearly visible on the snow surface. All the samples that were seen were collected unless the faeces were very severely degraded and rotting. An estimated 10% would have been missed in difficult to see areas such as in between tree roots or in very thick vegetation.

The faeces were collected and labeled with the following information: date, location within the compound, and an estimate of the amount of degradation the sample had undergone. Each sample was rated with a code from A-D with A being reserved for samples where the actual defecation had been seen. Samples that looked to have no degradation (i.e. within several hours to a day) received a B and D samples were already decomposing and showed very little form. Samples with a C rating were somewhere in-between the B and D ratings and were probably more than 1 day old. The time of year

also had an effect on the rate of degradation and therefore samples collected in the winter generally were in better conditions and received higher ratings. Some faecal samples were also immediately dried in a homemade dryer. The dryer was a plywood box 55 x 55 x 84 cm with 5 screen shelves and four 100W light bulbs mounted in the bottom with a small 120V cooling fan for circulation. Dried samples were pulverized using a Waring Commercial blender, screened with a 425 µm sieve and stored until extraction at -20°C.

Blood from CCWR wolves that died during the study period (May 1996-May 1998) was collected during necropsies. Approximately 200 µl of blood was added to 1000 µl lysis buffer (1.25% SDS, 0.3 M Tris-HCl pH 9.0, 0.1M EDTA and 5% sucrose) and stored at 4°C until the extractions were performed. In Quebec, blood was collected from 20 free-ranging animals while they were immobilized for the fitting of radio collars for a study by the Ministère Environnement et Faune du Quebec. Samples were stored in the same manner described above. Field crews working on the project also collected unidentified wolf scats from the same area.

Extraction Methods

Several extraction methods were performed on the blood and faecal samples.

1. Phenol/Chloroform Extractions

DNA was extracted from blood using a standard phenol/chloroform extraction protocol (Sambrook *et al.* 1989). One-hundred µl of the blood/lysis buffer mixture was added to an Eppendorf tube containing 100 µl lysis buffer. The mixture was incubated at 60°C for 30 min before 200 µl of phenol and 50 µl chloroform were added. Some samples were also digested with 10 µl proteinase K (10 mg/ml, 60°C, 30 min) after the

initial incubation step. After vortexing for 30 s the tubes were spun for 10 min (10 000g) in a Fisher Scientific microcentrifuge and the upper layer was transferred to a new tube containing 200 µl chloroform/isoamyl (24:1) alcohol. After agitation and centrifugation the supernatant was transferred to a new tube containing 200 µl chloroform, agitated, centrifuged (1 min, 10 000g) and the upper layer added to 200 µl isoproponal. The sample was agitated for 30 s and left overnight at -20°C before centrifugation for 30 min (10 000g). The isopropanal was poured off the DNA pellet and the pellet was washed with 100 µl 95% ethanol. After the samples were left to dry overnight the DNA was resuspended in 50 µl TE pH 7.0 buffer or distilled H_2O. Fresh, frozen and dried faeces were all extracted using the same phenol/chloroform extraction described above.

2. Guanidinimum Isothiocyanate Extractions

Dried, fresh and frozen faeces were extracted using a guanidinimum isothiocyanate and glass milk method (Boom *et al.* 1990). Approximately 100mg of frozen faeces were shaved into 1 ml of L6 extraction buffer (L6: 5 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH 8.0 and 1.3% Triton X-100) and incubated under constant agitation for 1-24 hours. The sample was then centrifuged (10 min, 10 000g) and the supernatant transferred to a new tube with 50 µl glass milk and 300 µl of the same L6 extraction buffer.

The glass milk was prepared as follows. Silica (6 g silicon dioxide, SiO_2; Sigma Chemical Co.) was mixed with H_2O to a total volume of 50 ml in a 50 ml graduated cylinder. The sediment was allowed to settle at room temperature for 24 hours and the

top 43 ml portion was discarded. Another sedimentation step was performed for a 5 hour period and 44 ml of the supernatant was discarded. The remaining silica/H_2O mixture was corrected to pH 2 with approximately 60 µl HCl (32%, wt/vol) and stored at room temperature in the dark.

After a 10 min incubation in the glass milk/L6 buffer, the sample was centrifuged for 10 s (10,000 g), the supernatant was discarded, and the pelleted glass was washed twice with washing buffer (L2: 5 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH 8.0), twice with 70% ethanol, and once with acetone before drying at 56°C for 10 min. The DNA was eluted with 100 µl TE pH 8.0 or H_2O at 56 °C for 10 min. DNA was also eluted with H_2O adjusted to pH 8 or pH 10 to see if greater yields could be produced. Some samples were also recentrifuged before the supernatant was transferred to a new tube leaving the glass pellet behind, while some were stored with the glass present. All extractions were stored at -20 °C.

3. Potato Flour and Purgene[c] Extractions

Frozen faecal samples were also purified using a modification of a method described by Deuter *et al.* (1995) which uses potato flour as an absorption matrix to remove bile acids which can inhibit the PCR. Approximately 200mg of frozen faeces was added to 600 µl of stool lysis buffer (SLB: 500 mM Tris HCL pH 9.0, 50 mM EDTA, 10 mM NaCl) and homogenized with a 1 ml pipette tip melted into a pestle. One fourth of the homogenate was added to 200 µl SLP containing 100 mg potato flour and mixed vigorously. The sample was centrifuged at 10 000 g for 10 min and 150 µl of the supernatant was transferred to a new tube containing 0.375 mg proteinase K. After

incubation at 60°C for 30 min the sample was extracted using the phenol/chloroform or the GuSCN method described earlier.

Faeces were also extracted using a commercial Purgene© kit as described by the manufacturer.

Yield Gels and Dilution of Stocks

Agarose gels (1%; 0.5g agarose, 50 ml 0.5X TBE, 1 µl EtBr) were used to separate 3 µl of the extracted blood or faeces which was mixed with 3 µl load/stop dye (0.25% bromophenol blue, 40% sucrose, 100mM EDTA) and 4 µl H_2O before loading. The gels were run for one hour at 90v and visualized with UV light. Each gel contained 1 µg of Hind III molecular weight marker (Promega) to estimate the quality and quantity of DNA present. All stocks containing the silica pellet were recentrifuged before use or dilution to eliminate suspended glass from entering the PCR mix.

Stocks from DNA extracted from blood were diluted with H_2O to a working strength of 400pg/µl if the amount of extracted DNA could be estimated using the known amounts in each fragment of the Hind III marker. Estimating the amount of DNA extracted from faeces was not possible because of the unknown amount of non-target DNA present so a dilution of 1:10 or 1:30 was tried. If no product was produced stocks were used without dilution or diluted in series until 1:1000 to try to produce a product. Once dilutions were mixed they were stored at 4°C until use (for 3 weeks or less).

PCR Conditions

Seven canid specific microsatelite primers (see Table 1) were selected from Ostrander *et al.* (1993) based on their variability in other studies and obtained from Cybersyn. The polymerase chain reaction (PCR) was used to amplify DNA present in the blood and faecal extractions. Amplification of the extracted DNA was performed in a 96 well programmable thermal cycler (MJ Research PCT-100) for 20-44 cycles.

Each reaction contained 1.3-4.8 µl template DNA, 10-80 mM Tris-HCl (pH 8.3-9.0), 0-20mM $(NH_4)_2SO_4$, 0-50 mM KCl, 0-6.0 mM $MgCl_2$, 0.15-0.2 mg/ml BSA, 0-0.1% Triton X100, 80 µM each dNTP, 0.25-5 µM of each primer and 0.5-1 units Taq DNA polymerase (Promega) in a total volume of 10-26 µl.

The PCR consisted of an initial denaturing step of 5 min at 95 °C followed by repeated cycles of denaturing for 30 s - 1 min at 91 °C, annealing for 30 s - 1 min at 55 - 64 °C and extension for 45 s at 74 °C. Some reactions were run using a stepdown PCR where an initial denaturing step of 5 min at 95 °C was followed by repeated cycles of denaturing for 1 min at 91 °C and annealing starting at 64 °C and ending at 55°C by a drop of 1°C each cycle, followed by a further 28 cycles with annealing at 55°C. Load/stop dye (3-6µl) was added to all reactions before separation in a gel or storage at 4°C or -20°C.

Gel Conditions and Visualization

The PCR products were separated and visualized in one of the following ways. Reactions were loaded into 2% agarose gels (1g agarose, 50 ml 0.5X TBE, 1 µl EtBr) and visualized with UV light or run through 4-12% polyacrylamide gels (15-20 cm long,

Table 1. Summary of the loci used and their primer sequences, annealing temperatures and the lengths of the alleles obtained during the study.

Locus	Primer sequence (5'-3')	T_a	Alleles	bp
377	ACG TCT TGA TGT ACA TTC CTG C	58, 60, 61,	A	145
	CCA CCC AGT CAC ACA ATC AG	63, 64	B	149
			C	151
			D	153
			E	155
			F	159
			G	161
250	TTA GTT AAC CCA GCT CCC CCA	58, 62, 63	A	128
	TCA CCC TGT TAG CTG CTC AA		B	130
			C	136
			D	138
			E	142
109	AAC TTT AAG CCA CAC TTC TGC A	60, 61, 63	A	142
	ACT TGC CTC TGG CTT TTA AGC		B	146
			C	150
123	AAC TGG CCA AAC ATA AAC ACG	58, 61, 63	A	140
	TTC ATT AAC CCT TTG CCC TG		B	144
			C	146
200	TTG ATC TGA ATA GTC CTC TGC G	57,63	A	118
	AGC AAC CCC TCC CAT TTA CT		B	126
173	ATC CAG GTC TGG AAT ACC C	59	A	98
	TCC TTT GAA TTA GCA CTT GGC		B	116
213	AAT ATG GGA GAG GAG AAG AGG G	57, 58	A	146
	ATG CTT CCT GGT AAG CAA TCA			
204	CAA AGT GCT GTG GCA GGT C	65, 68	A	196
	CGA GAG CAA CAT AGG CAT GA			

Note: T_a indicates annealing temperatures that produced product that was identifiable; alleles were lettered from smallest to largest starting with A using their base pair length.

0.4mm thick) in 1X Tris-borate buffer. Wells (N=15) were either cast in the gel or a 36 well shark's-tooth comb was inserted after casting. All gels had 1-5 lanes of PhiX174 DNA marker (Promega©) for size reference. Gels were either submerged in an EtBr bath (40 µl EtBr, 300 ml H_2O) for 10-20 min and photographed with Polaroid 667 film on a UV light source or stained with the following silver staining procedure modified from Tegelstrom (1986).

Gels were submerged in 0.1% cetyltrimethylammonium bromide (C-TAB). Next, 0.4 g silver nitrate and 0.04g of NaOH were each added to 2 ml distilled water in eppendorf tubes. At this time 10g $NaCO_3$ was added to 500 ml H_2O and left to dissolve with occasional agitation. After 15 min in the C-TAB the gels were transferred to distilled H_2O for 15 min and then to 0.3% ammonia. When the gels were placed in the ammonia the stain bath was prepared as follows. The silver nitrate/H_20 mixture prepared earlier was added to 248 ml distilled H_2O and agitated and then the NaOH/ H_20 mixture was added. Finally, 1 ml of 25% ammonia was mixed in and the stain was incubated at room temperature. After 15 min in the ammonia bath the gels were transferred to the stain bath and 270 µl of 37% formaldehyde was added to the $NaCO_3/H_2O$ mix to produce the developer. After 15-25 min in the stain bath the gels were transferred into the developer until dark brown/black bands appeared (5-20 min). The gel was fixed with one of the following three methods; 30 min in 2% glycerol, 20 min in 7.5% acetic acid or 5 min in Kodak© fixer (mixed as directed by the manufacturer) followed by a 5 min rinse in tap water at room temperature. Gels were either photographed with Polaroid© 667

film or dried onto a heavy paper backing in a model 543 Biorad gel dryer at 80 °C for 1-8 hours.

Microsatellite alleles were scored visually by comparison to adjacent lanes and the PhiX174 DNA marker (Promega). Wolves whose samples had interpretable results across several gels were used as benchmarks and their alleles were sized using the PhiX174 DNA marker. Other alleles in the same gels were then compared to the alleles of the benchmark individuals and whenever possible were sized by following the 2 base pair stutter that existed below the alleles.

Results

Five wolves in the CCWR pack died during the study period and extractions of blood from their carcasses with the phenol/chloroform method produced high yields of good quality DNA (Figure 1). Blood from 26 free-ranging wolves from Quebec was extracted following capture for another study and these samples produced yields similar to the CCWR wolves. Identified faeces were collected for all of the CCWR animals. Phenol/chloroform extractions of the faeces from the wolves produced smears with very little high molecular weight DNA. Most of the DNA was fragmented and less than 2 000 bp long.

When microsatellite amplification was attempted on the DNA extracted with phenol/chloroform from faeces no amplification was ever obtained. GuSCN extractions produced similar looking yields to the phenol/chloroform extraction with small amounts of high molecular weight DNA and fragments of all lengths down to less than 100 bp (see Figure 2). The residual glass powder that remained after the extraction fluoresced under UV light if loaded directly into a well and it was thought that some of the extracted DNA was remaining adhered to it. In case this was occurring, an attempt to free any adhered DNA was made by increasing the pH of the H_20 used to elute the DNA from pH 7 to pH 8 and pH 10. Unfortunately, this had no effect on the amount of DNA recovered. Even though the yield gels showed very fragmented DNA from the GuSCN extracted faeces, PCR's were attempted and showed some small quantities of product around the expected 100-300 bp length when separated on agarose. Reactions using DNA extracted

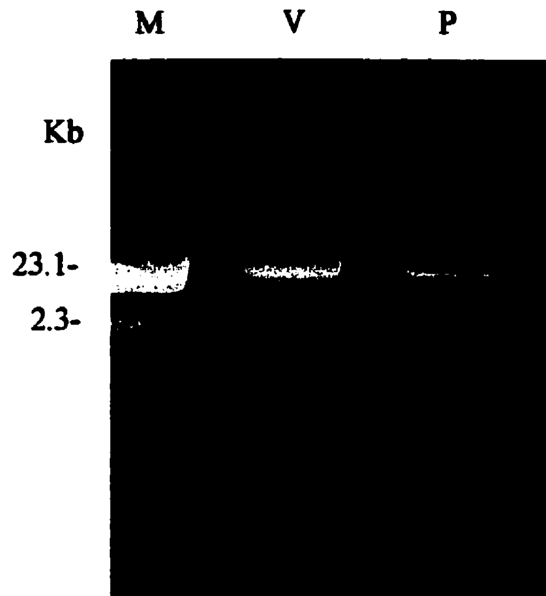

Figure 1. Chromosonal DNA recovered from the blood of two different animals using a standard phenol/chloroform extraction. Lane M, DNA marker lambda DNA digested with *Hin*dIII, lane V, Voochco and lane P, Pawnee. Three microlitres of the extracted DNA was loaded onto a 1% agarose gel.

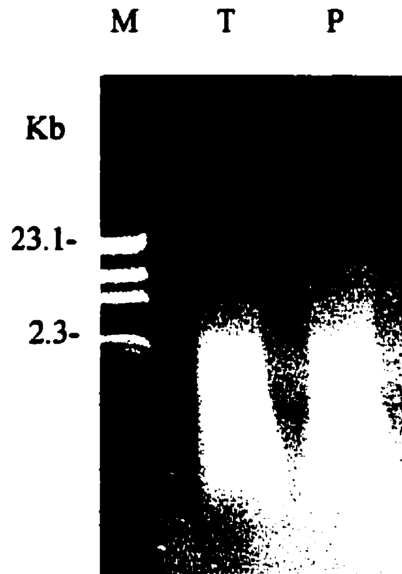

Figure 2. Chromosonal DNA recovered from the faeces of two animals using the guanidinium isothiocyanate and glass milk extraction. Lane M, DNA marker lambda DNA digested with *Hin*dIII, T, Tess and P, Pawnee. Three microlitres of the extracted DNA was run through a 1% agarose gel.

from faeces using the potato flour and Purgene© methods did not yield any PCR product; this was abandoned in favor of the GuSCN method.

Initially, 625 reactions were performed on blood or faeces samples and run through agarose or polyacrylamide gels. These first reactions were either stained with Etbr or silver stained and photographed. Seventy-five (12%) of these showed some product in the correct size range but due to the limitations of the EtBr staining and the small physical size of the photographs, the alleles were not distinguishable enough to score. Drying the polyacrylamide gels after they were silver stained produced the best image with the most discernible bands and 2 677 reactions were performed and run on these gels. Of them, 1 651 (62%) were completely unsuccessful where none of the reactions from a single PCR run produced any product.

Of the remaining 1 026 reactions where at least one of the reactions from the same day produced a product, 364 (35%) did not show any amplification at all and another 484 (47%) produced a product that was not interpretable for one of several reasons.

The main reason for a product not being interpretable was the presence of more than two bands near the expected allele length which made distinguishing the correct allele or alleles impossible. Microsatellites typically have one or more stutter bands that run shorter than the actual allele by the amount of the repeat, in this case two bases (Mockford, pers. comm. 1998). While these do give the reader a ladder-like marker that usually makes scoring easier when you can line up adjacent bands, it can also have the negative effect of masking alleles if the stutter is not distinguishable from the actual

allele. This caused some of the alleles to be uninterpretable. Bands that were diffuse and broad or were not separated enough on the gel and therefore spanned many base pairs were also often a problem. As well, in several cases the product could not be scored because the molecular weight marker was accidentally not loaded and the length of the product was therefore unknown. Furthermore, the products in some gels were not visually strong enough to identify alleles with confidence and were therefore not included in the scored results. Unfortunately, sometimes alleles that were strong enough to score were separated by several lanes with no product. This made comparison between them or the molecular weight marker impossible as comparison to an adjacent lane was not possible and the alleles were not scored.

Only the remaining 178 (17% of the reactions that yielded a product) had alleles that were considered interpretable. The annealing temperatures that produced interpretable results are shown in Table 1 along with the primer sequences and the alleles that were produced for each locus. Only products that were sharp enough to be unambiguous were scored and are shown in Table 2. An example of a reaction that was considered interpretable is shown in Figure 3.

<u>Locus 377</u>

Locus 377 had the most alleles of any locus with two alleles present in the CCWR pack and 5 in the Quebec animals with no alleles common to both populations. All of the 62 reactions that produced interpretable results had stutter bands present. Four of the eight CCWR wolves that were successfully genotyped were heterozygous with both the B and D alleles while three where homozygous for the B allele and one for

Table 2. Summary of the genotypes identified for wolves from the CCWR and Quebec.

Location	Animal	Loci: Number in parenthesis indicates the number of different reactions that produced the allele							
		377	250	109	123	200	173	213	204
CCWR	Pawnee	B D (7)	C E (3)	A A (1)		B B (4)	A A (1)	A A (2)	
	Xyla			A A (1)					
	Tess	D D (8)	D E (3)	A A (3)		A B (3)		A A (2)	
	Morgaine	B B (1)							
	Devil Child	B D (4)	C E (2)	A A (4)		A B (1)			
	Voochco	B B (8)	D E (4)	A A (2)		A A (3)	B B (1)	A A (2)	
	Galen	B D (2)				A B (1)			
	Homer	B B (1)							
	Ulysses	D B (1)				B B (1)			
Quebec	16				A A (1)				
	27			A A (1)	A A (1)				
	30	A E (1)		A A (1)	*				
	33			A B (1)	A A (1)				
	36	A F (3)		A B (1)	A A (1)				
	37	G G (4)	A B (1)	A B (2)	A A (1)				A A (2)
	39	E F (4)	A B (1)	A B (2)					
	42	C C (1)		A C (2)	A A (1)				
	43	G G (5)	A B (1)	A B (4)	A A (1)				
	44				B C (1)				
	45	G G (4)	B B (1)	A B (4)	A A (1)				
	46	F F (2)	B B (1)	A A (3)	B B (1)				
	47	A G (1)		A B (1)					
	48	G G (1)		A A (3)					
	49			A B (2)					
	50			A B (1)	A A (1)				
	51			A B (2)	A A (3)				
	52	C C (1)		A A (2)	A B (2)				
	53	C C (1)		A A (2)	*				
	54	C C (1)		C C (2)	*				

Note: Individuals that could not be assigned a genotype because of varying results from different reactions and were assigned a *. Repeats during the same run are only counted once.

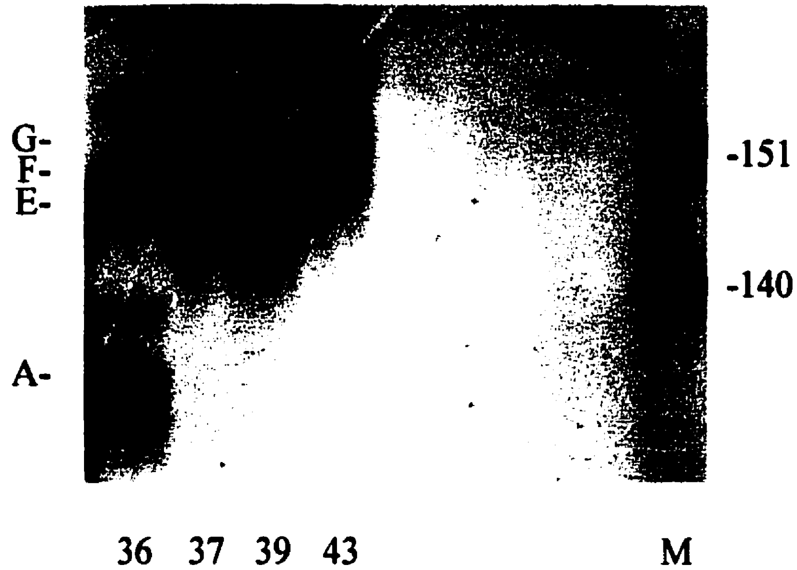

Figure 3. Microsatellite alleles (A, E, F, G) amplified from the blood of 4 Quebec wolves with primer 377. Products are run through 8% polyacrylamide gels and then silver stained. Lane M is PhiX 174 DNA marker. Note the mirror bands that appear 6-8 bases longer than the actual alleles and the 2 bp stutter that appears below each allele.

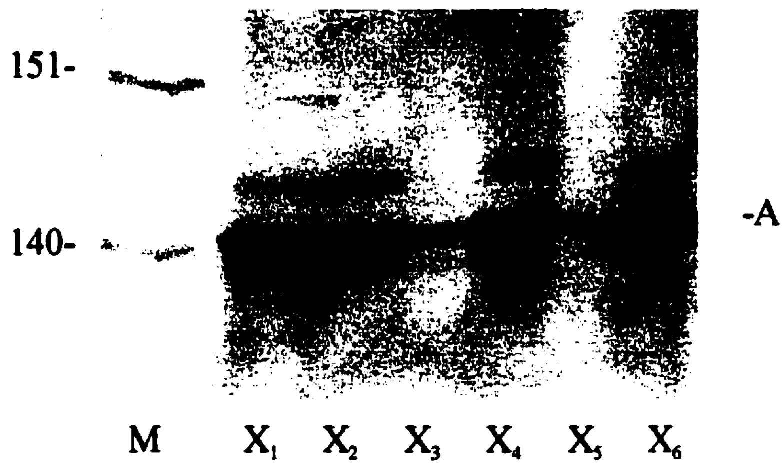

Figure 4. Microsatellite allele A amplified for locus 109 from six different extractions of the same scat sample from Xyla. Products are run through 8% polyacrylamide and then silver stained. Lane M is PhiX 174 DNA marker.

the D allele. Thirteen of the Quebec wolves were genotyped with four being heterozygous and nine homozygous.

Locus 250

Only 13 reactions with the CCWR animals produced identifiable alleles with stutter bands. The four CCWR animals scored were all heterozygous and they each had allele E, while two also had allele C and two had allele D. Two different alleles were seen in the five Quebec wolves with three of them heterozygous with the A and B alleles and two of them homozygous with only the B allele.

Some sets of reactions also produced bands that were sharp and distinguishable but where no stutter was present. All the reactions from the same run would either have or not have the stutter. Individuals' alleles ran at different lengths when the stutter was not present. The reactions without the stutter were not scored as alleles to keep consistency with all the other identifiable products.

Locus 109

Three different alleles were seen in the Quebec wolves and only one in the CCWR wolves. All the Quebec samples run more than once were consistent with each other with the exception of animal number 36. In one reaction using DNA from animal 36 alleles A and B are clearly visible where on another date only allele A is present. All of the CCWR wolves were homozygous for allele A. Four of the CCWR samples also showed bands at the allele length of allele B on 2 days but the bands never had any evidence of stutter and were therefore not considered as alleles.

Locus 123

No product for locus 123 was ever produced with any CCWR wolves, but product was produced for 13 of the Quebec wolves. Two wolves were heterozygous, one with alleles B C and one with A B while 10 were homozygous for allele A and one wolf was homozygous for allele B.

Locus 200

Only six of the CCWR wolves were genotyped for locus 200. Three were heterozygous for alleles A B, two were homozygous for allele B and one for allele A.

Locus 173

Two alleles were produced for locus 173 for two CCWR animals. Pawnee was homozygous for allele A and Voochco was homozygous for allele B.

Locus 213

Readable product was produced for only three CCWR individuals on two occasions. The wolves, Pawnee, Tess and Voochco, both had the same allele at 150 bp.

Locus 204

On one occasion a very sharp allele was seen for animal 37 from Quebec with a length of 196 bp. This allele was produced on the same day in two different PCR reactions at annealing temperatures of 65°C and 68°C. Strong product of the same length was produced on one other day with four Quebec individuals but was not interpretable as the bands were blended into one another and were not distinguishable.

Product from Faeces

While most of the alleles that were identifiable came from DNA extracted from blood, some faecal samples did produce results. In a test to see the reliability of the DNA extracted from faeces, six different extractions were performed from the same piece of faeces. The extracted DNA from Xyla's faeces produced the identical genotype for locus 109 from all six extractions (see Figure 4). Unfortunately repeating the same six extractions at other times failed to produce any interpretable products. Similar problems with replication occurred with samples from other individuals. One other reaction worked for locus 109 on Tess where three different dilutions of the same extraction from a faecal sample each produced a product that matched the product produced from DNA extracted from her blood. Although the dilutions of 1:100 and 1:500 did produce a weak product only the more concentrated 1:10 sample produced an identifiable one.

The greatest number of interpretable reactions from DNA extracted from faeces was obtained with locus 377. In one set of reactions, duplicate extractions from the same faeces matched identifiable product from blood for the individuals Pawnee, Tess and Voochco (see Figure 5). On the same day product was obtained from duplicate extractions from six other individuals but unfortunately were not identifiable due to stutter bands that were not distinguishable from the actual alleles. Reactions that were run using DNA from blood from two of the individuals were also impossible to score.

In another set of reactions using primer 377 two out of three matched blood/faeces sets produced identifiable product while the third was too weak to score reliably. In the same run faeces from three out of five individuals from whom a blood

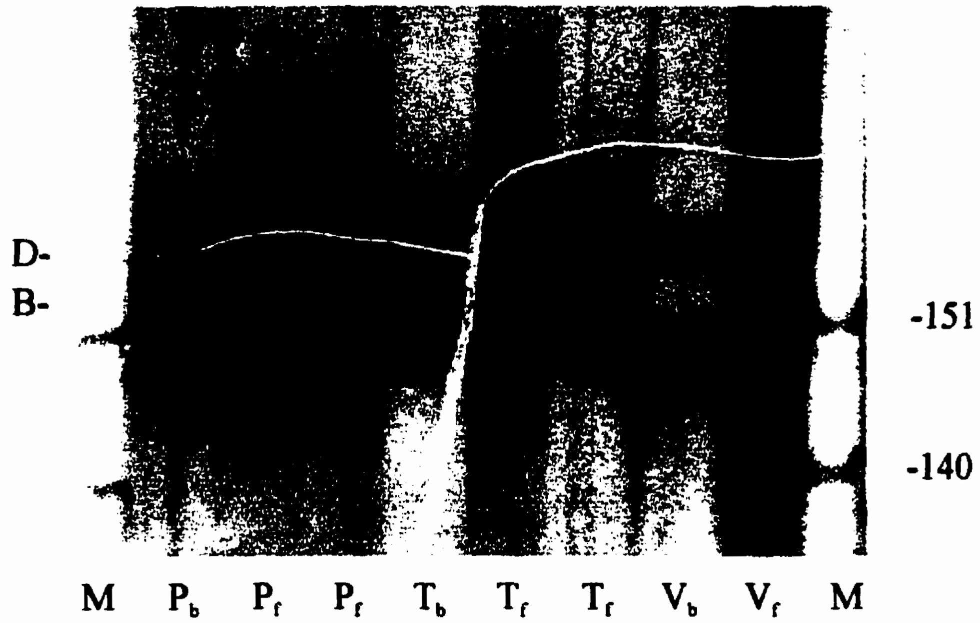

Figure 5. Microsatellite alleles for locus 377 amplified from DNA extracted from matched blood and faeces for 3 CCWR animals (P-Pawnee, T-Tess, V-Voochco). Subscript b indicates template was DNA from blood and subscript f indicates DNA was from faeces. Lane M is PhiX 174 and products are separated on 8% polyacrylamide.

sample was never obtained produced product good enough to score. In two other reactions alleles were scored from DNA recovered from Galen's faeces.

Galen was also genotyped for locus 200 with DNA extracted from his faeces.

Discussion

In this discussion I will evaluate my success in completing each of the objectives outlined in the introduction. While only one of the six was not achieved the others were all successful to some degree. Unfortunately, the overall aim of identifying the donor animal of collected faeces was never fully reached although the results demonstrated that with more refining of the technique this could be possible. The success of each of the stated objectives will now be discussed.

The first objective was to determine the most appropriate method of extracting DNA from faecal samples. The potato flour extraction and the commercial Puregene© kit were unsuccessful at recovering any DNA. The DNA that was obtained using a standard phenol/chloroform extraction was never successful in producing a PCR product and all of the above were abandoned in favor of the guanidinimum isothiocyanate and glass milk extraction. This extraction provided a relatively quick (under 2 hours) method of recovering DNA that was successful in producing alleles that were strong enough to score.

Unfortunately, the second objective of evaluating the amount of degradation that a scat can undergo before it is no longer possible to extract DNA that will amplify was not met due to the difficulties in getting the fresh samples to work consistently. In a related study that used DNA from seal scats, it was found that scats that were classified as 'slimy' and therefore had not weathered for long, were more likely to yield products than those samples with a 'sandy' classification (Reed *et al.* 1997). Since the 'slimy' samples only made up 18% of their samples, and alleles were produced from all

classifications, they did not find a clear break at any one texture class. Future work with wolf faeces would need to further address this question, as at some point degradation would make amplification impossible. Different storage methods other than freezing and drying should also be tried in an attempt to address this problem.

The third objective of this study was to see if microsatellites could be successfully scored in a small lab without the equipment and resources of a large lab with access to radioisotope labeling. It was obvious after a short time that working with agarose gels was not an option because it was not possible to visualize products in the range of 100-200 bp down to the 2 bp resolution that was required and a gel with better separation was needed.

Polyacrylamide gels used at 8% gave better resolution than the agarose gels but staining with ethidium bromide only showed products with a large amount of DNA. If enough volume was loaded into the gel for the staining to be effective the lanes were overloaded and bands spanned far too great a distance to make scoring down to two bases possible. Gels ranging in polyacrylamide concentration up to 12% did not improve sharpness as the higher concentration resulted in a longer run time with more diffusion. Lower polyacrylamide percentages resulted in even less separation in the 100-200 bp area and were therefore less effective than the 8% gels. The use of gradient gels was considered but was decided against as they only increase the readability of a gel if the products span several hundred base pairs (Sambrook 1989).

Silver staining was used to attempt to improve the sensitivity of the visualization and the resulting gel was photographed while still wet on a white piece of paper with

daylight and Polaroid© 667 film. This detected DNA at quantities down to 100 pg which was far more sensitive than the ethidium bromide staining which is only sensitive to around 10 ng.

The interpretation of the image was still limited by the small size of the photograph, which made small differences impossible to distinguish. Drying the gels on blotting paper allowed interpretation at full size which was a great improvement but caused the background of the gel to turn to a dark yellow colour if the gel was fixed with glycerol or acetic acid. Although many of these gels were interpretable, a fixation step in Kodak fixer instead of in glycerol or acetic acid and a rinse in distilled water, eliminated the colour shift. With this step the background stayed clear to light grey which provided a better contrast with the dark brown to black bands.

It was found that even greater sensitivity was achieved if the gels were left in the silver nitrate stain for 25 min instead of the 20 min that was initially tried. Very weak products that were not visible with a 20 min stain became visible with the extra stain time. Staining times greater than 25 min did not increase the sensitivity.

Changing to polyacrylimide gels allowed the separation of DNA down to the resolution that was required. This increased resolution did not come without extra cost when compared to the ease of working with agarose gels, as many problems were initially encountered in the running and staining of the polyacrylamide gels. Initially, it was difficult to get the glass plates to seal well enough in a vertical position to be able to pour the polyacrylamide gels without having the gel leak out before it was polymerized.

This was solved by casting the gels in a horizontal position with the bottom and sides of the gel sealed with a bead of agarose that was injected with a 1 ml pipette.

At first, 2.5cm wells were cast into 18 cm gels leaving only 15.5cm of gel for separation of the product which was not long enough to resolve the 2 base pair difference that was required. To gain more separation of the alleles the plates were changed to 20 cm, the maximum length possible in the tank that was available, and a shark's tooth comb was used. The shark's tooth comb gained another 2.5cm of running length by eliminating the wells at the top of the gel. The shark's tooth comb also allowed the loading of up to 36 lanes of product and eliminated the space in between lanes that was present if wells were cast in the gel itself. The one drawback to the shark's tooth comb was that lanes of product or molecular weight marker were sometimes not contained and leaked into adjoining lanes. Loading the gel as quickly as possible and working from one side to the other so that each well had adjacent wells filled quickly, creating an equal pressure between lanes, helped to eliminate this problem.

Finding the correct amount of product to load was another problem. If not enough product was run there would not be sufficient DNA to stain and if too much product was run the bands would be thick and not sharp enough to be scored. Both of these problems occurred and were only overcome by trial and error. When using the shark's tooth comb a maximum of approximately 10 µl could be run so 6 µl of the product was loaded, run and stained and then if needed the sample was rerun with the volume adjusted as required. Another problem that sometimes occurred with the gels was that the insertion of the comb caused the top surface of the gel to become deflected from

a straight line. If this was severe enough it caused problems in scoring the gel if stutter below the alleles could not be followed from one lane to another.

Marginal separation of the alleles was another major problem. The alleles usually needed to be run to the bottom of the gel without running them off the end. If they were not near the bottom of the gel they were usually difficult to score and even sometimes when they were, the separation was not clear if the bands were diffuse or thick. This caused many reactions to be uninterpretable and may only have been overcome with a longer gel tank or different primers. Tri and tetra-nucleotide microsatellite primers for canids where the repeats are 3 and 4 bases apart respectively, would be a very desirable development for small labs attempting this type of work. The difference in separation may make the difference in making a greater portion of the reactions interpretable, given the restricted resolution of the techniques described here.

The thickness of the gel also affected the readability of the product. Gels used for separating radioisotopic products are usually only 0.25 mm thick. This was initially tried but the problem was that the lanes had to be overloaded to get enough product to be visible when stained and they produced a broad diffuse band. Increasing the thickness of the gel to 0.4 mm helped overcome this problem by increasing the surface area available for the product to run through. This was evident because if you looked at a gel carefully you could percieve depth to the band because of the thickness of the gel. As a result, it also made visual separation of the bands more difficult if you were not looking at an allele perpendicular to the gel's surface. This was overcome by drying the gels after they

were stained and fixed which concentrated the band by decreasing the thickness of the gel to virtually nothing on the paper backing.

Gels were run under many different conditions but running 20 cm gels at 275 volts for 3 hours without circulating cooling water in the tank produced the best results. Gels were initially run with circulating tap water behind the glass plates that cooled the gel. Running the gels without this cooling caused the gels to be run in less time with even less diffusion in the bands. If the gels were run at voltages of 300 V or greater without cooling then the gel would overheat and cause the bands to smear together. If the cooling was run at these high voltages to overcome some of the heating problems then the outer glass plate often cracked between the top buffer tank and the cooling reservoir, presumably from the temperature differential between the two.

The fourth objective of amplyifing DNA from both blood and faeces was successful even though the process never became consistent enough to be considered routine. Amplification from faecal samples was most successful in the earlier stages of the study but this may have been an artifact of working with it more at this time. When it was realized that amplification from blood was also sporadic the effort was concentrated on making the blood work consistently and faecal samples were used less as the study progressed. Even so, alleles were scored for many of the individuals of both the CCWR pack and the collected samples from the free-ranging wolves in Quebec. Any blood and faeces samples from the same individual that were amplified successfully also produced identical genotypes which indicates the microsatellites obtained from the faeces were accurate.

One problem with many of the actual PCR products was the presence of excess bands around the alleles that made distinguishing the true alleles impossible. Although other researchers reported successful scoring at annealing temperatures of 50°C - 58°C (Forbes and Boyd 1996, García-Moreno *et al.* 1996) interpretable products in this study were usually only obtained at temperatures higher than they reported. Superfluous bands sometimes even occurred in the high range of the successful annealing temperatures and attempts were made at minimizing these extra bands by adjusting PCR buffer mixes. Since superfluous bands are commonly observed when scoring radiolabeled microsatellites (Mockford S, pers. comm. 1998) completely eliminating them in this study would be an unrealistic goal.

Nested primers, which may have helped eliminate superfluous bands, especially in DNA recovered from faeces, were not available for these microsatellites. Using nested primers involves using two different primer sets (Roux 1995). A first PCR reaction is run which amplifies a long product that includes the area of the DNA that contains the allele that is targeted. This helps to concentrate the portion of the DNA that contains the allele of interest. The product from the first PCR is then used as template DNA in a second reaction using the second set of primers. This second set of primers produces the alleles of interest from a source that has a high concentration of the area of the DNA containing the allele with little contamination from the rest of the genome.

A second set of bands that ran 6-14 bases longer than the actual allele was also often present in many of the reactions. The difference between the actual allele and the "mirror" allele was consistent for all the reactions with the same primer. This mirror

allele must not be confused with the stutter bands described earlier which always run at 2 bp intervals shorter than the allele. For example, if a mirror allele was present in a set of reactions for primer 377 it was always 6 or 8 bases longer. This did not usually cause a problem as the shorter set of the two was always scored. However, there were some cases where the true alleles, mirror alleles, and the stutter bands all overlapped and scoring then became impossible.

The mirror bands may be an artifact of the silver staining and the fact that the DNA strands may be denaturing in the gel and running at a slightly different length. With silver staining, both would be visible. In most microsatellite studies only one of the two primers is γ-P^{32} end-labeled and the product is run in a denaturing gel (Sambrook *et al.* 1989). This results in only one of the strands being visible and even if the second strand runs shorter or longer it would never be seen. Intentionally, denaturing gels were not used in this study as they would have exaggerated the problem of seeing both strands of DNA. However, there is a possibility that denaturing occurred during gel running.

Although complete genotyping for all the wolves was not possible many comparisons to other studies were possible, thus fulfilling the fifth objective. The largest barrier encountered when attempting to genotype all the individuals in the CCWR and Québec groups was getting reactions to consistently produce readable products. Reactions using identical protocols and reagents on subsequent days sometimes produced results one day and not on the other. Why this occurred is still not understood. One possibility is that the storage of the extraction material and the storage of extracted DNA may have both been inadequate to prevent degradation of the DNA past the point of

successful amplification. Deuter *et al.* (1995) report that the two major problems associated with using DNA extracted from faeces are degradation during periods of storage and the presence of copurified excremental substances such as bile salts and bilirubin. They found that, depending on the efficiency of the extraction methods in removing these substances, up to 80% of the DNA degraded after storage at -20°C. Using potato flour as an absorption matrix improved the stability of the extracted DNA in their study but unfortunately did not produce any PCR product when it was tried in this study. Wasser *et al.* (1997) evaluated storage procedures for scat samples and found that drying faeces in silica beads was an effective preservative but the same procedure was not possible within the scope of this study.

Other researchers report difficulty in genotyping from faeces. When working with excrement samples from the European brown bear Kohn *et al.* (1995) report that repeated extractions from the same sample were needed to account for non-amplifying reactions. Even when performing six parallel extractions on each of 12 samples only eight of the samples produced a product in at least one of the six extracts. The authors speculate that this difference occurs from uneven shedding of cells from the intestinal lining.

In the same study the authors evaluated the length of amplifiable DNA present in extractions from faeces. Amplifications on 12 individuals of 398 bp, 295 bp and 141 bp portions of the mitochondrial control regions were attempted on DNA extracted from excrement, liver and hair. While all the 12 samples amplified at all 3 lengths from the liver and hair samples only one excremental sample amplified products up to the 398 bp size. Of the remaining 11 samples, five produced product up to 295 bp, two amplified

only the 141 bp product and four did not produce any amplification at all. These results clearly show a bias toward a greater success by targeting shorter sequences. Since several hundred copies of mitochondrial DNA are present in each cell (Kohn *et al.* 1995) attempting amplification of single copy nuclear DNA would presumably be even more difficult.

In a study using DNA recovered from seal scats Reed *et al.* (1997) were able to separate harbour and grey seal scats using a microsatellite locus with non-overlapping alleles between the two species. In their study they began with 173 samples and evaluated 18 primer sets to determine if successful amplification from the faeces was possible. They found that only five were successful while nine providied no positive identification and the remaining four may have been useful only after further primer optimization. In this study it was also apparent that some of the loci were more successful than others, yet optimization of the primers was not an option due to resources available in the lab. The seal researchers were able to genotype 82 of the 173 samples with data for 4-5 loci enabling them to find a total of 67 unique genotypes. They hypothesized that the samples that were not successful were either too old, washed by a tide which could have removed sloughed off cells or have a diet bias where a food item was influencing the molecular techniques. Although in this study all the faecal samples that were used were collected fresh one possible inhibitor to the successful amplification of the samples in this study could have been food items as hypothesized above.

Comparison of the alleles found in this study and those in published studies is only performed with caution because all the reported wolf microsatellite studies used γ-

P^{32} end labeled primers and M13 sequence as a marker which allows exact sizing of the product down to a single base. However, some very close matches were observed. In Forbes and Boyd's (1996) study of the genetic structure of wolf populations in the Rocky Mountains, the reported alleles were all within 6 bases of the same length. For example, the greatest number of alleles was at locus 377 where they report 9 alleles with lengths of 146, 148, 156, 158, 160, 162, 164, 166, and 172 bp. In this study locus 377 also had the greatest diversity of alleles with the CCWR wolves having lengths of 149 and 153 and the wolves in Quebec with 145, 151, 155, 159 and 161. Reported alleles for locus 109 at 143, 145, 147, 149, 151, 153 and 155 also matched alleles found in this study with lengths of 142, 146 and 150. The other loci that were used in both studies, 250, 123 and 204 were also overlapping or at most were 6 bases apart. While very small sample sizes from this project prevents any further analysis between the two studies the similarities between the two strongly suggest that the alleles scored with silver staining were accurate.

The sixth objective of confirming the suspected pack pedigree (from observation of matings and lactating females) of the CCWR wolves was only possible in a limited manor due to the incomplete genotyping for many of the individuals at many of the loci. Even if all the individuals had been genotyped with the primers that were used, additional loci may have also been required, as two of the loci that did work were homozygous in all individuals scored. The genotypes that were obtained do all match suspected pedigrees (see Appendix 1). For example, the alpha wolves for most of the study period were Pawnee and Galen. Their offspring, Morgaine, Devil Child and

Ulysses each had one or both of their parent's alleles. Galen is also suspected to be an offspring of Pawnee and shares one or both alleles with her. Tess, also an offspring of Pawnee, shares one of her alleles at each of the five loci that worked. The other wolves, Xyla and Voochco are litter mates of Galen and Pawnee respectively and each share at least one allele with their siblings.

In conclusion, amplifiable DNA was recovered from wolf faeces using a guanidinimum isothiocyanate and glass milk extraction method. Microsatellite genotypes were obtained for many of the CCWR and Quebec timber wolves using silver staining, a non-radioactive technique which is available to small labs. Genotypes obtained from DNA extracted from blood and faeces collected from the same individuals always matched indicating accurate amplification of the DNA from faeces. Both the close overlap of alleles of this study and alleles reported in another study and the matching of the suspected pedigree of the CCWR pack with obtained genotypes, strongly suggest the scoring was accurate. While the identification of unknown scats was never attempted, the results shown in this study strongly advocate the refining of this technique for use in future studies.

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Appendix 1. Suspected pedigree of the Canadian Centre for Wolf Research wolves from behavioural observations. The sex of each wolf is indicated by the subscript letter.