A GENETIC ANALYSIS OF THE EASTERN TIMBER WOLF

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EASTERN TIMBER WOLF

By

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2001)   McMASTER UNIVESITY
(Biology)   Hamilton, Ontario

TITLE: A Genetic Analysis of the Eastern Timber Wolf.

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SUPERVISOR: Professor Bradley N. White

NUMBER OF PAGES: xiv,173

Abstract

While studying packs of the eastern timber wolf in Algonquin Provincial Park in
Ontario, DNA profiles at 8 microsatellite loci and the mitochondrial control region were
found to be similar to those of the red wolf, *C. rufus*. Based on this it was suggested that
both the red wolf and the eastern timber wolf have a common origin, evolving in North
America, with the coyote diverging from them 150,000 – 300,000 years ago and with
neither having any recent connection with the gray wolf that evolved in Eurasia. It was
further proposed, that the eastern timber wolf retain its original species designation of *C.
lycaon* instead of the present status of a subspecies of the gray wolf.

Four "types" or "races" of wolves have been previously described in Ontario. Using
DNA profiles, assignment tests identified four groups, which were typified by animals in
Algonquin Provincial Park, Pukaskwa National Park, Frontenac Axis and those north of
Lake Superior. The tests indicate that Frontenac animals are hybrids between the western
coyote and *C. lycaon* and represent the eastern coyote. Pukaskwa maintains a small wolf
population, which is genetically closer to the gray wolves of the Northwest Territories
than the surrounding *C. lycaon*. These may represent an isolated remnant population of
the original "Ontario type" (*C. lupus*). Animals north of Lake Superior were identified as
C. lycaon, but represent products of hybridization between *C. lycaon* and *C. lupus*.

Currently within Ontario, Algonquin Park contains the largest protected area of the
eastern timber wolf. DNA profiles, including Y-linked microsatellite loci were used to
establish maternity, paternity and kin relationships for 102 animals from 24 packs over a

12-year period. A complex pack structure was identified. A pack is not composed simply of an unrelated breeding pair and their offspring and subordinates appear to enter pack systems through adoption, pack splitting, dispersal and immigration. Relatively high genetic structuring was found between the Park animals and the "Tweed" wolves to the southeast suggesting introgression of coyote genetic material is not a present concern to the integrity of park animals. Evidence of gene flow with animals to the west, northeast and northwest coupled with the high genetic diversity, suggest that the Park animals are not an island population, but the southern part of a larger metapopulation of *C. lycaon*.

Increased interest in the relationship of the red and eastern wolves led to the investigation of a gene in the major histocompatibility complex. Allelic variation in the exon 2 region of the DLA-DQA1 locus was analysed for gray wolves, red wolves, the eastern timber wolf and the western coyote. Twelve alleles were identified, seven of which were previously characterized in dogs. Non-synonomous nucleotide substitutions was 3.0 times higher than the synonomous changes, indicative of strong positive selection. These data provide baselines for the determination of allele frequencies and their distribution across the geographical range of the four species in North America.

The results in this thesis have sparked numerous debates with respect to the protection of the wolves in Algonquin Provincial Park and reintroduction of wolves into Northeastern United States. The data support the idea that the *C. lycaon* population in Ontario is relatively large, numbering in the thousands rather than the hundreds. Concern for the conservation of wolves in Ontario should be directed at the declining numbers of gray wolves present in Ontario.

Acknowledgements

It has been a long journey and as with any of life's lessons I have learned many things not only about myself but about the strength and determination that each of us possess inside our hearts. These lessons in life would not have been possible and sometimes not even bearable without the many people who have contributed to my journey.

I would first like to thank members of my supervisory and examining committee for their interest in my work, of whom I especially owe a great deal of gratitude to Dr. Bradley White, my supervisor, whose patience, support and guidance have kept me sane throughout these last few years.

In addition my sincere gratitude toward the numerous people who participated in sample collection over the years including David Pennock, Dennis Voigt, Will Waddell, Robert Chambers, Paul Paquet, Gloria Goulet, Dean Cluff, T. L. Hillis, Frank Mallory and Hillary Sears. A special thanks to John and Mary Theberge, without whose data and sample collection from Algonquin Provincial Park over the last 12 years, this thesis would not have been possible.

Further, I would like to extend my appreciation to my many colleagues over the years; Carole Yauk, Brent Murray, Sobia Malik, Diana Polley, Chris Schultz, Ihor Boszko, John Wang, Ruth Waldrick, Carla Shaw, and many of the fourth year students

who have helped along the way. A special thanks to Paul Wilson who helped me through my thesis in countless ways and taught me virtually all that I know.

Thanks to my many friends, who put up with me through the years without whom there wouldn't have been as many fond memories. Tina, Kristina, Brenda, Cathy, Pearl and Tanya, you gave me hope when there was none and laughter when all I could do was cry.

I would like to thank my family whose love, support, patience and encouragement made this all possible. And finally I would like to dedicate this thesis to my mother, who taught me strength, courage, commitment and hope above all. She was my mother, my friend and my hero. Without her love and encouragement throughout all aspects of my life I would not be the person I am today.

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Scope of Thesis

This thesis focused on characterizing the wolf species present in Ontario and their relationship to each other and western coyotes. The distribution and population size of these species and hybrids are important with respect to wildlife policy and management in Ontario. In order to characterize the species, the evolutionary relationship of wolves in eastern North America was addressed in the second chapter. In the last century wolf taxonomy was the centre of a number of debates but none so controversial as the nature and origin of the red wolf. Both Nowak and Wayne have debated for years on whether the red wolf was a hybrid between a western coyote and gray wolf or a separate North American-evolved wolf. Fossil records, morphological differences and recently genetics have been used to help resolve as well as fuel some of these debates. This thesis added substantially to this discussion by focusing on the eastern North American canid species and hybrids and their relationship to each other.

In Ontario the wolves of Algonquin Provincial Park have been at the center of many debates with respect to wolf conservation. They are currently a major tourist attraction and have become the focus of the two sides of the debate. After centuries of hatred and fear for the wolf, much of society is becoming increasingly aware of the wolf as an important part of our wildlife heritage. With this increased interest a movement for the protection of the Algonquin wolf has developed, however there are many groups, especially hunters and farmers that are still hesitant to accept the wolf as part of the natural landscape.

Concerns regarding the long-term conservation of wolves in Algonquin Provincial Park have been recently raised due to the increasing number of human caused wolf mortalities outside the protected Park. Many of the issues have been brought to the surface because of results from radio tracking and ecological studies done by John and Mary Theberge in Algonquin Provincial Park. To address the concerns of the long-term conservation of the wolf, the Honorable J.C. Snobelen, Minister of Natural Resources formed the Algonquin Wolf Advisory Group in 1998. Some of the concerns that need to be addressed before recommendations can be implemented are. 1. Are wolves declining in the Park because of human caused mortality, when wolves migrate to winter deer yards outside Algonquin Park, and when the territories of the wolf packs overlap Park boundaries? 2. Are the wolves in the Park threatened by interbreeding with coyotes dispersing into the Park? and 3. Are wolves in the Park genetically unique and restricted to the Park?

Many of these questions are addressed in Chapters 3 and 4 of this thesis by assessing wolves in Algonquin and their relationship to surrounding populations in Ontario and Quebec. Chapter three characterizes Ontario wolves and attempts to unravel the Canis soup. Most of this Canis soup is a result of changes in the ecosystem over the last two centuries, which has led to the northern movement of white-tailed deer and the expansion of western coyotes into eastern North America. Questions regarding the historic presence of a larger gray wolf in the Park and its relationship to the smaller eastern timber wolf now present are discussed in detail. Chapter four focuses more on the finer scale of the issues in the Park and the interactions of the wolves with the surrounding canis

populations. Threats to the wolf with respect to its long-term persistence in Algonquin Provincial Park are addressed.

The initial studies used neutral genetic markers to address many of the questions raised. These included mitochondrial DNA sequences and alleles at autosomal and Y-chromosome microsatellite loci. With the increased interest in the origin of the Algonquin wolf and the differences in prey base between the different North American canis species, we used a selective marker for further investigation. The final chapter therefore looks at alleles of a functional gene in the major histocompatibility complex in both coyote and wolf species. Differences in genetic profiles at a selective marker can impact the future management of the different canis species in North America.

Chapter 1

GENERAL INTRODUCTION

Wolf and coyote systematics and evolution

Based on the fossil records, it has been hypothesized that an ancestral wolf-like

canid inhabited North America approximately 2 million years ago (Nowak 1979; Nowak

1995). It has been suggested that about this time, individuals of this group emigrated to

Eurasia and evolved into the present gray wolf, *Canis lupus*, which then migrated back to

North America via the Bering land bridge during the Pleistocene ice age, approximately

300,000 years ago (Nowak 1979; Kurten and Anderson 1980) initially inhabiting the

Arctic Islands. The species was able to adapt to the numerous habitat types and move

south to occupy taiga, hardwood, mixed and softwood areas. By this time wolves were

thought to occupy most of North America, except for deserts and tropical rain forests. The

wide variation in color, size and weight of North American wolves was noted by many

early authors and was the foundation on which earlier taxonomic classifications of wolves

was based.

Wolf taxonomy of North America

Early morphological studies (Goldman 1944) led to the recognition of 24 gray wolf

subspecies in North America (Hall and Kelson 1959). Based on skull measurements and

proposed Pleistocene refugia, this was reduced to five *C. lupus* subspecies by Nowak in 1983; *C. lupus arctos*; *C. lupus occidentalis, C. lupus nubilus*; *C. lupus baileyi* and *C. lupus lycaon*. The gray wolf however, was not the only wolf to receive attention from taxonomists and conservationists. The origin of the red wolf, *C. rufus*, has been debated by a number of groups (Nowak 1995; Roy et al. 1996). Nowak (1983) has suggested that in contrast to the gray wolf, the red wolf is a North American evolved canid (Figure 1.1A). This theory, which suggests that the red wolf is a separate species was challenged by Wayne and his colleagues based on genetic data. They suggested that the red wolf is a hybrid resulting from the interbreeding of gray wolves and coyotes (*Canis latrans*) (Wayne and Jenks 1991; Roy et al. 1994; Roy et al. 1996) (Figure 1.1B). This idea was largely based on the absence of mtDNA and microsatellite sequences in red wolf samples that were not of either gray wolf or coyote descent (Wayne and Jenks 1991; Roy et al. 1994; Roy et al. 1996).

Wolf taxonomy of eastern North America

Earlier classifications of wolves in eastern North America by Miller (1912), recognized 5 species; *C. lycaon* (eastern Canada); *C. floridanus* (Florida), *C. frustror* (junction of Neosho and Arkansas Rivers), *C. lupus var rufus* (Texas) and *C. mexicanus* (southern Mexico). Both *C. lupus var rufus* and *C. floridanus* were later recognized as subspecies of the red wolf, *C. rufus floridanus* and *C. rufus rufus* respectively. Pockock (1935) recognized many of the species identified by Miller (1912) as subspecies of the gray wolf, *C. lupus*, but maintained the eastern Canadian wolf as *C. lycaon*. Following a

Figure 1.1: Two models for the evolution of canids in North America. The progenitor of

wolves and coyotes is indicated at the top. It is generally accepted that divergence from

this ancestor occurred 1-2 million years ago (YA), when the progenitor of *C. lupus*

migrated to Eurasia. (A) Represents Nowak's model of the evolution of North American

wolves based on skull measurements. (B) Wayne's model of the evolution of North

American wolves based on genetic data. In both hypotheses the eastern timber wolf is

represented as a gray wolf subspecies.
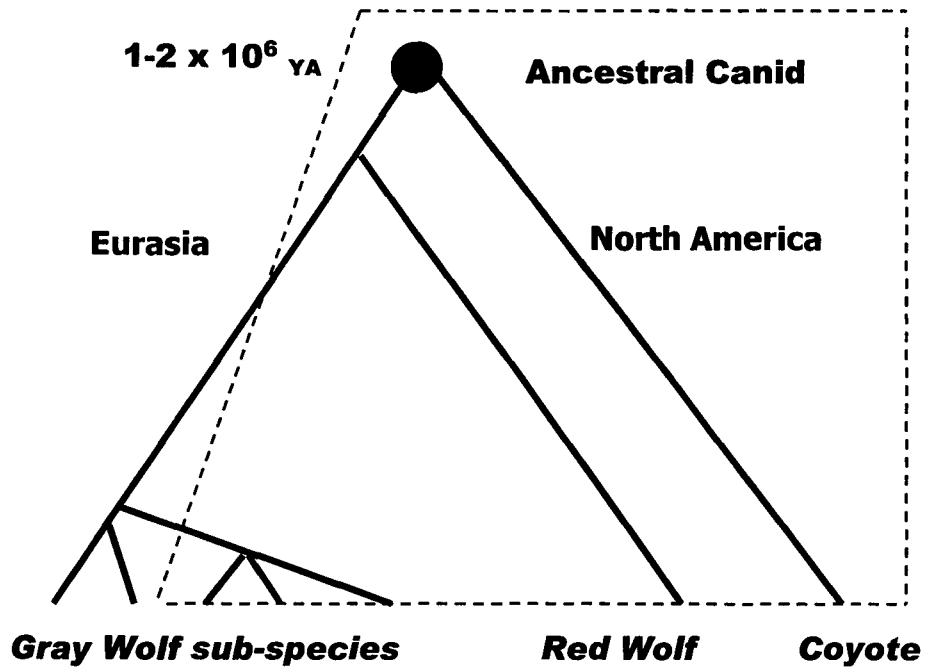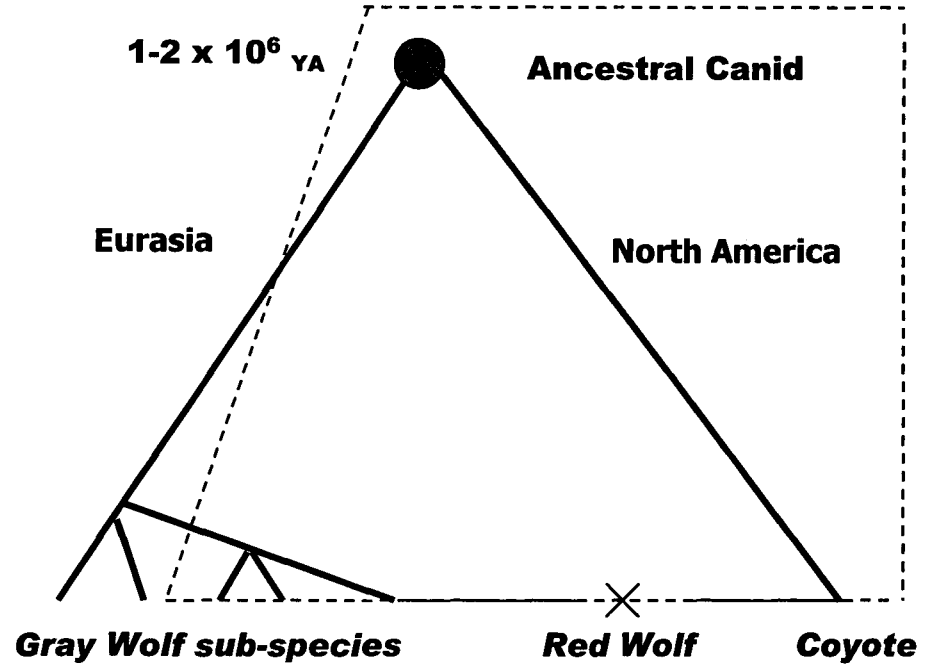
A

1-2 x 10^6 YA

Ancestral Canid

Eurasia

North America

Gray Wolf sub-species

Red Wolf

Coyote

B

1-2 x 10^6 YA

Ancestral Canid

Eurasia

North America

Gray Wolf sub-species

Red Wolf

Coyote

number of revisions, Young and Goldman (1944) produced a comprehensive treatment that considered the eastern Canadian wolf a subspecies of the gray wolf (*C. l. lycaon*). Throughout the subsequent taxonomic debates on wolves in North America, few changes to the wolf types in eastern North America have been made. In a final taxonomic review by Nowak (1995) five gray wolf subspecies and one red wolf species were recognized in North America. In eastern North America, *C. l. lycaon* was identified in the north and *C. rufus* in the south (although numbers are now limited to those individuals involved in the captive breeding program and release sites). There is no geographic boundary separating the two wolves raising issues to whether there are indeed two species. The lack of geographic barriers or barriers to gene flow between species or subspecies of wolves continues to be a concern in taxonomic debates in North America.

One area of specific concern with respect to geographic boundaries is in the province of Ontario. Various numbers of gray wolf subspecies have been identified in Ontario over the years. In 1944, Goldman recognized *C. l. lycaon* as the only gray wolf subspecies in Ontario, while Hall and Kelson (1959) included *C. l. hudsonicus* along the coastal area of Hudson Bay. Following this revision, Nowak (1983) accepted the presence of a second gray wolf subspecies, but claimed that *C. l. hudsonicus* should be classified as *C. l. occidentalis*. Finally in 1995 Nowak suggested grouping North American wolves into 5 subspecies based on the Pleistocene refugia. Three of these were identified in Ontario based on skull measurements, *C. l. occidentalis*, *C. l. nubilus* and *C. l. lycaon* (Nowak 1995). Based on this proposed distribution, most of Ontario was inhabited by *C. l. nubilus*, a subspecies originally assigned to the central Plains of the US.

It is evident that throughout the taxonomic debates there has been little consideration of the barriers to gene flow that originally must have been present to cause and maintain the differences among the "types" or subspecies. The Pleistocene ice sheets clearly had a major impact on the distribution of wolves and their ungulate prey in North America, as did the arrival of the Europeans in the 17th century. Human impacts such as deforestation, farming, trapping and bounty hunting, extirpated wolves throughout most of the continent providing opportunities for the expansion of the coyote and the subsequent breakdown of the reproductive barriers between coyotes and eastern wolves.

Predator-Prey relationships in Ontario

Gray wolves were thought to have occupied all of Ontario prior to European colonization (Bates 1958) preying primarily on larger ungulates such as elk (*Cervus elaphus*), moose (*Alces alces*) and caribou (*Rangifer tarandus*). Following the establishment of the Hudson Bay Company in 1670 and intense logging in the late 1800s, significant declines in ungulates and wolves occurred in south and central Ontario (Peterson 1955; Franzmann and Schwartz 1998). One region significantly affected after 1840 was Algonquin Provincial Park, which underwent intense logging and wolf control programs. The reduction in gray wolves and deforestation probably facilitated the movement of white-tailed deer and the smaller eastern timber wolves from the south at the end of the 19th century.

At the beginning of the 20th century western coyotes entered southern Ontario and probably hybridized with residual pockets of eastern wolves to form the eastern coyote

which rapidly spread further eastward (Moore and Parker 1992; Wilson et al. in Prep) to occupy the Frontenac Axis and Magnetewan regions. As a result, these areas contain a diverse range of sizes of wolves and coyotes for which the term "canis soup" was coined. Kolenosky and Standfield (1975) recognized four "types" or "races" of wolves in Ontario. This complexity has been attributed to wolf hybridization with the coyotes, *C. latrans*, which began colonizing Ontario in the early 1900s (Kolenosky and Standfield 1975). Although a similar expansion of coyotes into northwestern North America occurred there has been no similar formation of "canis soup" in areas inhabited by western wolves. This raised the issue as to what is different between eastern and western North America.

The controversy surrounding the taxonomic and evolutionary issues surrounding wolves in eastern North American is a complex one, with many questions still not addressed. Is the eastern timber wolf a separate species from gray wolves, red wolves and coyotes? Which of these species are in Ontario and specifically in Algonquin Provincial Park? How many wolves are in Ontario and can the wolves in Algonquin Park be maintained in a changing ecosystem? Genetic markers provide useful information to address these questions.

Genetic Markers

Genetic markers have been used to address issues of taxonomy and conservation for some time (Avise et al. 1987; Wayne 1993; Ellegren et al. 1996; Villa et al. 1999; Murray et al. 1999). Both neutral and functional markers within the mitochondrial and nuclear DNA have been used. The variation at these markers makes them useful for identifying

species, populations or individuals. Some of these markers include polymorphic regions

of the mitochondrial DNA, which are maternally inherited without recombination. The

control region is one of the most variable regions within the mitochondrial DNA and is a

useful marker for elucidating phylogenetic relationships among closely related taxa as

well as assisting in parentage analysis. The substitution rate for the mammalian control

region is 1-2% per 100,000 years (Stewart and Barker 1994). The mitochondrial genome

of a species may remain in a hybrid population long after one species has gone extinct in

a given region, revealing vestiges of past hybridization events (Lehman et al. 1991). By

comparing sequences in present day individuals, questions regarding family trees as well

as population movements can be addressed. The one drawback is that the mitochondrial

DNA is only passed from mother to offspring, therefore only addressing female

movement. Recently more attention has been given to the Y-chromosome, which can be

used to construct paternal lineages.

The most informative markers on the Y-chromosome are microsatellite loci. These

polymorphic nuclear markers have become one of the major tools used to assess variation

at both the individual and population level. Microsatellites are composed of simple

tandem repeats (2-5bp) and have a substitution rate as high as 10^{-4} per generation (Tautz

1989). Their rapid evolutionary rate (Tautz 1989) makes them useful markers for

distinguishing closely related species as well estimating parentage and relatedness among

individuals (Double et al. 1997; Blouin et al. 1996; Prodohl et al. 1998). In addition to

microsatellite markers located on the Y-chromosome, other microsatellite loci are found

throughout the nuclear genome and are bi-parentally inherited. These loci provide

information on the transmission of genetic information from both the maternal and paternal line. As a result, DNA profiles obtained using these loci are important with regard to genetic differentiation, hybridization and relatedness.

Another category of genetic markers, which are useful in ecological studies are nuclear functional genes such as those in the Major Histocompatibility Complex (MHC). MHC genes are members of the immunoglobin superfamily and represent some of the most polymorphic and thoroughly investigated genes in the vertebrate genome (Klein 1986). Genes of the MHC encode glycoproteins that bind foreign antigens (peptides) and present them to circulating T-lymphocytes to initiate an appropriate immune response. MHC genes are best known in humans and mice where they have been classified into three evolutionarily related groups, Class I, Class II and Class III (Klein 1986; Campbell and Trowsdale 1993). The complex spans several millions of base pairs (about 4Mbp in humans, which is approximately equivalent to the size of the *Escherichia coli* genome). Balancing selection is thought to drive the high level of polymorphism observed at the MHC loci. It is suggested that this selection may be linked to a pathogen-based model (Klein 1987, Potts and Slev 1995), primarily due to the involvement of MHC genes in the immune response. By possessing two MHC alleles at each locus, an individuals' ability to initiate an immune response is increased because of the wide number of antigens it can bind. An individual that lacks a specific MHC allele may be unable to initiate an immune response to a specific invading parasite or pathogen. Both overdominance (Nei 1987) and frequency dependent selection may play roles in maintaining this diversity (Takahata and Nei 1990). In heterozygous advantage / overdominance, a heterozygote has a higher

fitness than a homozygote because it can respond to a wider range of parasites. In frequency-dependent selection, alleles at low frequency have a selective advantage because pathogens will not have had enough time to evolve the ability to infect host cells carrying a new allele. Diversity can also be maintained through reproductive mechanisms involving maternal fetal interactions (Clark and Kirby 1966; Gill 1982) as well as mate choice (Yamazaki et al. 1988; Potts et al. 1991; Wedekind et al. 1995). Studies reveal that spontaneous abortions occur more often when MHC alleles are shared between parents and that females can detect males that differed at the MHC through odour.

Generally it is the positive selection at the MHC region that is of importance and this can be deduced through analysis of the peptide binding region. This region also referred to as the "antigen recognition site" is found in hyper variable regions identified in the exon 2 of many of the MHC loci. Comparison of nonsynonymous and synonymous substitutions at the PBR for both Class I and Class II loci show significantly greater number of nonsynonymous substitutions (Hughes and Nei 1988, 1989).

Many of these genetic markers can be used to identify relationships between individuals and populations. Genetic information in conjunction with ecological data is very important in completely understanding population dynamics over time and space.

Algonquin Provincial Park and the Wolf Advisory Group (AWAG)

After centuries of hatred towards the wolf, public attitudes have been slowly changing. Today much of society regards the wolf "….as an important part of our wildlife heritage" (AWAG 2001). This change in attitude has recently drawn much

attention to wolves in Algonquin Provincial Park. Recent concern regarding the role of

Algonquin Park as a protected area for wolves and the long-term persistence of its wolves

has sparked many debates. Much of these issues stem from the intense radio tracking and

ecological studies in the Park over the last 12 years by John and Mary Theberge and their

students from the University of Waterloo. Recent evidence suggesting a decline in

Algonquin wolf numbers, stimulated action from the Ministry of Natural Resources. In

1998, the Honourable John C. Snobelen, announced the establishment of the Algonquin

Wolf Advisory Group. The task of this group is to assess the current status of Algonquin

Park wolves and provide recommendations for an Adaptive Management Plan to ensure

the long-term conservation of wolves in the Park. Before the recommendations are

implemented a number of questions should be addressed. 1. Are wolves declining in the

Park because of human caused mortality, when wolves migrate to winter deeryards

outside Algonquin Park, and when the territories of the wolf packs overlap Park

boundaries? 2. Are the wolves in the Park threatened by interbreeding with coyotes

dispersing into the Park? and 3. Are wolves in the Park genetically unique and restricted

to the Park?

Research Objectives

This thesis attempts to address some of the questions raised by the Algonquin Wolf

Advisory Group. However before local questions regarding Algonquin Provincial Park

can be addressed, the evolutionary relationships of eastern North American canis species

needed to be investigated. This study expands on the on going debate of the origin of the

gray wolf subspecies, *Canis lupus lycaon* and the red wolf, *Canis rufus*. The taxonomic status is crucial for the management of wolves in Algonquin Provincial Park. Characterizing different geographic wolf and coyote populations in Ontario will enhance our knowledge of what species are present in Ontario and the potential conservation risks associated with hybridization between wolves and coyotes. Genetic information gathered in this thesis will be useful in future management decisions by the Ontario Ministry of Natural Resources.

The two major goals of this thesis are: 1) to identify the evolutionary relationship of wolves and coyotes in North America using functional and non functional genetic markers and 2) to characterize and assess the relationship of animals within Algonquin Provincial Park to those in the surrounding populations of wolves and coyotes.

The arrangement of the following chapters is as follows: Chapter two analyzes evidence for a common evolutionary history of the eastern timber wolf and the red wolf independent of the gray wolf; Chapter three characterizes wolves and coyotes across Ontario; Chapter four describes the genetic relationship between packs of the eastern timber wolf, *Canis lycaon* in Algonquin Provincial Park and their relationship to surrounding animals; and, Chapter five compares the MHC DQA1 allelic variation in three eastern North American canis species , followed by a general discussion.

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Preface

This chapter was written with co-authors and has been published in a peer-reviewed journal and is therefore a self contained manuscript with an individual reference section.

Chapter 2: DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf.

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Status: Canadian Journal of Zoology. 78:12. (2000).

Contribution: Microsatellite data collection done by S.K.G., J.N.M. and A.G.G. Mitochondrial data collection done by S.K.G and I.D.L. Data analysis done by S.K.G. and P.J.W. Writing of this paper was done by P.J.W., S.K.G. and B.N.W. Research was conducted under the supervision and guidance of B.N.W.

Chapter 2

DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf

ABSTRACT

The origin and taxonomy of the red wolf (*Canis rufus)* has been the subject of considerable debate and it has been suggested that it is a recently formed taxon as a result of hybridisation between the coyote and gray wolf. Like the red wolf, the eastern Canadian wolf has been characterised as a small "deer-eating" wolf that hybridises with coyotes. While studying the population of eastern Canadian wolves in Algonquin Provincial Park we recognised similarities to the red wolf based on DNA profiles at eight microsatellite loci. We examined whether this relationship was due to similar levels of introgressed coyote genetic material by comparing the microsatellite alleles with other North American populations of wolves and coyotes. These analyses indicated that it was not coyote genetic material that led to the close genetic affinity of red wolves and eastern Canadian wolves. We then examined the control region of the mitochondrial DNA and confirmed the presence of coyote sequences in both. However, we also found sequences in both that were 150,000-300,000 years divergent from sequences found in coyotes. None of the red wolves or eastern Canadian wolf samples from the 1960s contained gray wolf (*C. lupus*) mtDNA sequences. The data are not consistent with the hypothesis that the eastern Canadian wolf is a sub-species of gray wolf, as it is presently designated. We suggest both the red wolf and eastern Canadian wolf evolved in North America sharing a

common lineage with the coyote until 150,000-300,000 years ago. We propose that it retain its original species designation of *C. lycaon*.

INTRODUCTION

The origin of the red wolf, *Canis rufus*, has been the subject of considerable debate and controversy. Nowak (1979; 1995) proposed that the species evolved in North America from a wolf-like canid representing a transitional form between a coyote-like ancestor and the gray wolf (*C. lupus*) that evolved in Eurasia. Contrary to this hypothesis Wayne and Jenks (1991) and Roy et al. (1994, 1996) has suggested that *C. rufus* is not a valid species but the result of recent extensive hybridisation between *C. lupus* and coyotes (*C. latrans*) in the south central U.S. The taxonomic designation of *C. rufus* together with all North American canids has been fluid in this century ranging from less distinct than a sub-species, eg. *C. lupus* var. *rufus*, to its present species status (Brewster and Fritts 1995). There is general agreement that the red wolf hybridizes with the coyote.

The eastern Canadian wolf, *C. l. lycaon*, like the red wolf, has been the subject of several taxonomic treatments that have moved it from species status, *C. lycaon*, to its presently accepted status as a gray wolf sub-species (Brewster and Fritts 1995). Since the late 1700s, eastern North American wolves were described as among the smallest on the continent (Goldman 1944), long before any documented arrival of coyotes (*C. latrans*) in the 1900s. As with the red wolf, there is general agreement *C. l. lycaon* readily hybridizes with coyotes and studies of mitochondrial DNA have shown hybridisation

between wolf populations east of Minnesota and coyotes (Lehman et al. 1991, Wayne and Lehman 1992).

Wolf and coyote populations have been further compared using microsatellite loci (Roy et al. 1994); all coyote populations are closely related, whereas gray wolf populations representing different sub-species of *C. lupus* are more divergent. "Hybridizing wolf" populations in Minnesota and southern Quebec were genetically most similar to each other and then to captive red wolves. The original interpretation of these relationships was that "hybridizing wolves" of southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994).

The cause of wolf/coyote hybridisation has been attributed to the destruction of forested habitat and the increased expansion of coyotes in the last 90 years (Wayne and Lehman 1992). While these are clearly important factors, the introgression of coyote mtDNA and nuclear DNA into wolf populations appears limited to the eastern portion of North America. The hybrid zone that has been identified based on mtDNA and microsatellite DNA markers has not been assessed with respect to the sub-species of *C. lupus* that is involved (Lehman et al. 1991, Wayne and Lehman 1992, Roy et al. 1994, Nowak 1995). The proposed sub-species distribution of Nowak (1995) shows that the boundary of the hybrid zone corresponds closely to the historical distribution of the eastern Canadian wolf, *C. l. lycaon*. The absence of any introgression of coyote DNA into western wolf populations sympatric with coyotes, such as those in Alberta (Roy et al. 1994, Pilgrim et al. 1998) and Alaska (Thurber and Peterson 1991, Roy et al. 1994)

suggests that only the eastern wolves, *C. l. lycaon* and *C. rufus*, readily hybridize with coyotes.

While studying a population of the eastern Canadian wolf, *C. l. lycaon*, from Algonquin Provincial Park we found a surprisingly close relationship with the red wolf based on allele frequencies at microsatellite loci. Although both wolves are known to hybridize with coyotes, we prefomed several analyses to determine if it was introgressed coyote genetic material that led to their close affinity. We further examined mitochondrial control region sequences from captive red wolves, from coyote samples and from wolf teeth samples collected in Algonquin Park and elsewhere in Ontario during the 1960s. These represent wolves that had contact with coyotes for a period of less than 30 years. They are the best available natural sample set of eastern Canadian wolves to detect representative eastern Canadian wolf mtDNA. In this paper we test two alternative hypotheses that the red wolf/eastern Canadian wolf are hybrids of coyotes and gray wolves or that these wolves both derived independently of gray wolves in North America.

MATERIALS & METHODS

Samples and DNA Extraction

Eastern Canadian wolves, representing the putative gray wolf sub-species *C. l. lycaon*, were sampled from Algonquin Provincial Park and surrounding area from 1960-1965 (n=19) and 1985-1996 (n=49). *Canis rufus* samples from the captive red wolf breeding program (n=60) were also analysed. Texas coyotes (n=24) were used to

represent *C. latrans*. Gray wolves, *C. lupus*, were sampled from the Northwest

Territories (n=67). DNA was extracted by methods described in Guglich et al. (1994)

from frozen organ samples (liver, heart, kidney, or muscle) or from whole blood obtained

by venipuncture of individuals that were live trapped and released. DNA from the

captive red wolf program, Texas coyotes and historic teeth collected in Ontario during the

1960s was extracted following a modified Qiagen (Qiagen) extraction protocol using the

lysis buffer described in Guglich et al. (1994).

Microsatellite Analysis

Ten microsatellite loci (Roy et al. 1994; 1996; Ostrander et al. 1993) were amplified

using 4.6 pmol γ^{33}P T4 polynucleotide kinase (Boehringer-Mannheim) end labeled

primer ATP in a total reaction volume of 10μl per tube using 25ng of genomic DNA, 200

μM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, unlabelled primer (0.2 mM), 1.0 μg

of Bovine Serum Albumin (BSA) (BRL) and 0.5 units of *Taq* polymerase (BRL).

Products were amplified under the following conditions: 94°C for 5 min., 55-65°C for 30

sec., 72°C for 15 sec. 1 cycle; 94°C for 15 sec., 55°C for 30 sec., 72°C for 15 sec. 30

cycles; 94°C for 15 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were then

mixed with an equal volume of formamide loading buffer and were heated at 95°C for 5

minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control

sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size

markers for the microsatellite alleles.

Control region Sequencing and Sequence Analysis

The following primers were used to amplify the control region of the mitochondrial DNA.

Primer 1   5'-GAA GCT CTT GCT CCA CCA ATC-3' (Pilgrim et al. 1998)

Primer 2   5'-GGG CCC GGA GCG AGA AGA GGG AC-3'

The control region was amplified in a total reaction volume of 20μl per tube using 25ng of genomic DNA, 200 μM dNTPs, 1x amplification buffer, 2.0 mM $MgCl_2$, primers 1 and 2 (0.2 mM) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min., 55°C for 30 sec., 72°C for 30 sec. 1 cycle; 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec. 35 cycles; 94°C for 30 sec., 55°C for 30 sec., 72°C for 2 min. 1 cycle. Products were re-amplified and purified through QIAquick (Qiagen) for DNA sequencing using dye-terminator cycle sequencing using an ABI Prism 373 DNA Sequencer (MOBIX, McMaster University).

A previously described method (Pilgrim et al. 1998) for distinguishing *C. lupus* mtDNA from *C. latrans* was used to identify the presence or absence of gray wolf mtDNA within the historic teeth samples based on a 4 base pair difference between gray wolves and coyotes.

Genetic Analysis

We analyzed allele frequencies at 8 loci among the Algonquin Park and red wolf populations and compared them with the other North American populations of wolves and coyotes (Roy et al. 1994; 1996). Microsatellite alleles were assigned based on size in

Roy et al. (1996). Nei's genetic distance (1972) was calculated using the programs SEQBOOT, GENDIST and NEIGHBOR in the computer program PHYLIP (Felsenstein 1993).

An individual index (I_I) was calculated from the DNA profile of each animal using the following equation: $\Sigma \log (p_A/p_B)$, where p_A and p_B are the allele frequencies of a specific allele from population A and B, respectively. If an allele was absent from one of the populations, an allele frequency of one allele in the population (sample size) was used. This LOD score value assesses the origin of the alleles in each animal based on a ratio of the frequencies from two populations. If there are similar allele frequencies in both populations then the I_I values of individuals from both populations would follow a distribution around 0. An increasing positive score indicates an individual originated from population A and a decreasing negative score indicates an individual originated from population B.

A Probability of Identity (POI) measure (Paetkau and Strobeck 1994, Waser and Strobeck 1998) was also calculated to assess whether an individual's genotype was from one of two source populations. The probability of an individual's genotype using the allele frequencies of one source population is summed over all loci. The same calculations are made with respect to the second putative population. The log of the two values for each individual's genotype based on the two source population's allele frequencies are plotted to produce a scatterplot to assess the population with which the individual has the greatest likelihood of affiliation.

A minimum spanning tree was generated based on data provided by the program MINSPNET (Excoffier, 1992). The phylogenetic relationships of canid mtDNA haplotypes were generated using a neighbor-joining tree with sequence divergence using the program MEGA [Kumar, S. Tamura, K. Nei, N. MEGA: Molecular Evolutionary Genetic Analysis 1.01 (Pennsylvania State University, University Park, PA, 1993)].

RESULTS

The neighbor-joining analysis of genetic distances showed an unexpectedly close relationship among Algonquin Park animals, the red wolf, Minnesota wolves and the southern Quebec wolves (Fig. 2.1). To evaluate whether this was because "hybridizing wolves" of southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994) we determined DNA profiles from captive red wolves and other populations of gray wolves and Texas coyotes. The same relationship between eastern Canadian wolves and captive red wolves was observed when they were compared to gray wolves and Texas coyotes (Fig. 2.2). In this comparison, the interpretation that eastern Canadian wolves and red wolves shared similar levels of coyote introgression did not seem consistent with the genetic distance between the red wolf and the Texas coyotes, which were the geographically closest coyote source population for the red wolf. The genetic similarity between red wolves and eastern Canadian wolves was not heavily influenced by the introgression of coyote genetic material; alleles that were prevalent in Texas and other coyote populations (Roy et al. 1994) were absent or present at very low frequency in red wolves (Table 2.1).

Figure 2.1. Neighbor-joining tree of Nei's genetic distances for allele frequencies from eight microsatellite loci. With the exception of the Algonquin Provincial Park population, the source of the allele frequencies are from Roy et al. (1994, 1996). Two of ten dinucleotide microsatellite loci, i.e. cxx 344 and 213, from Roy et al. (1994, 1996) were excluded based on our observation of the presence of 1 base pair allele differences not found previously. As a result of the number of alleles differing by one base pair at these two loci, we excluded them from the analysis. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstrap re-samplings of the data, Algonquin and captive red wolves were grouped together in 72.4% of trees.

Jackal

Southern Quebec

Minnesota Wolf

Algonquin Provincial Park

Red Wolf

Minnesota Coyote

Alberta Coyote

Maine Coyote

Alberta Wolf

Northwest Territories

Northern Quebec

52.8%

72.4%

88.1%

55.9%

0.1
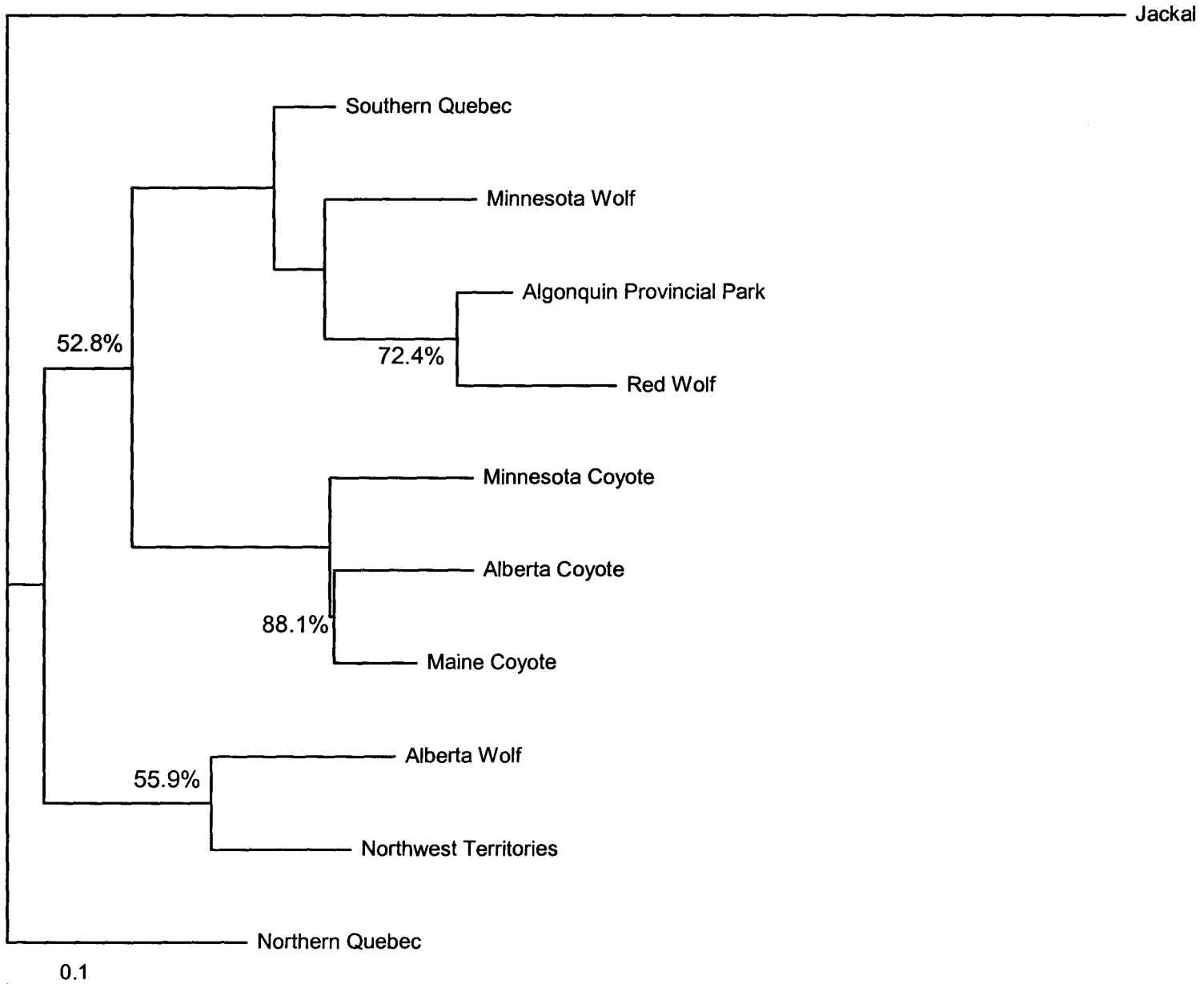
Figure 2.2. Neighbor-joining tree of Nei's genetic distances (1972) for allele frequencies from eight microsatellite loci for Eastern Canadian wolves, gray wolf populations and a Texas coyote population. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstraps re-sampling the data the Algonquin Park and captive red wolf population were grouped together in 67.8% trees. The neighbor-joining tree gave an approximation of the genetic relationship among these populations and alternative topologies are possible.

Texas Coyote

Algonquin Provincial Park

67.8%

89..2%

Red Wolf

Pukaskwa National Park

81.3%

Northwest Territories

50.8%

Riding Mountain National Park

0.1
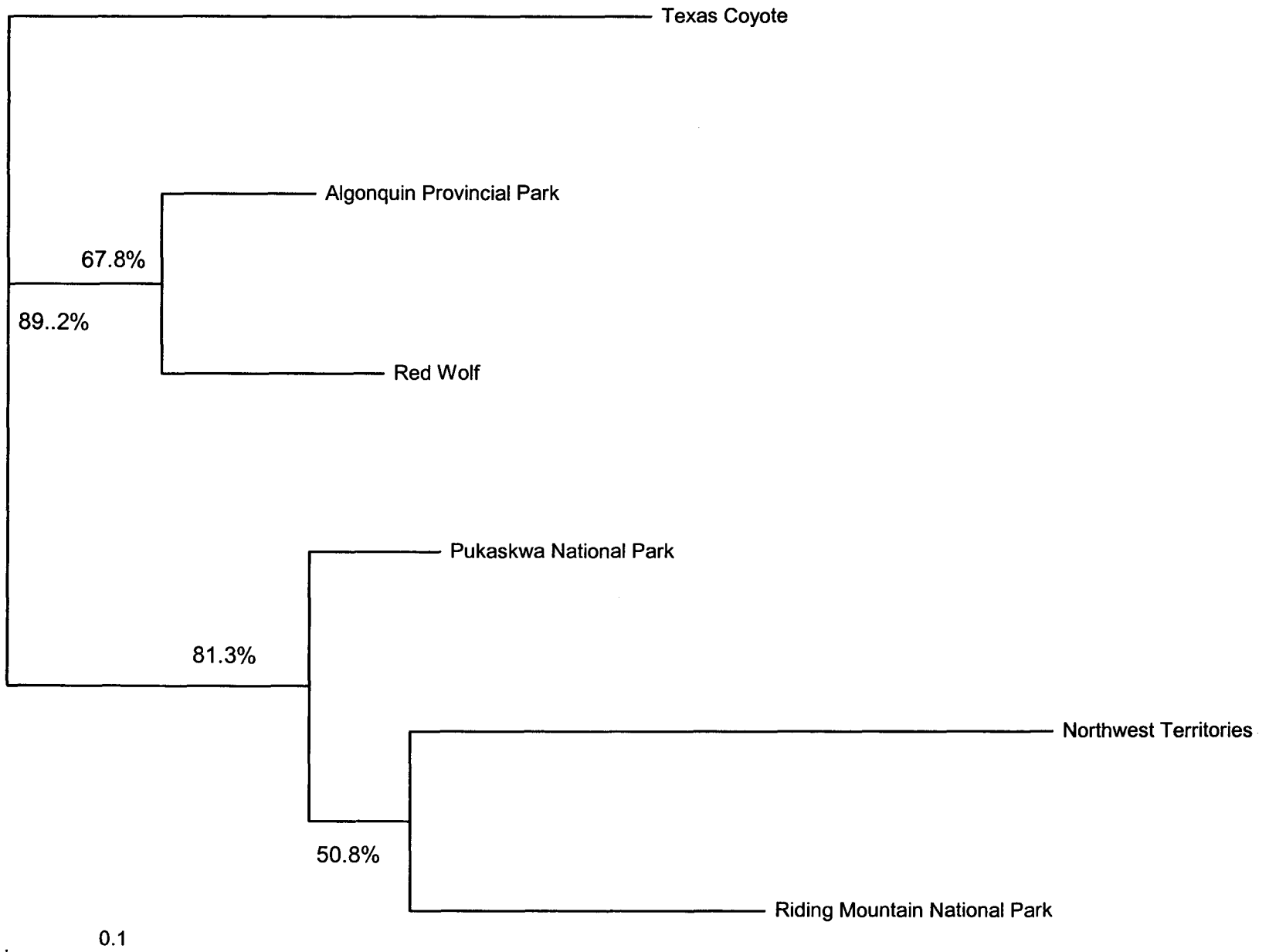
Table 2.1. Alleles prevalent in Texas coyotes and other coyote populations that are absent or present at low frequency among captive red wolves. Loci and allele designations and the first red wolf column have been previously described (Roy et al. 1996).

Locus	Allele	Texas coyote	Red wolf (Roy et al. 1996)	Red wolf (this study)
Cxx 225	B	0.239	0.000	0.000
Cxx 225	C	0.500	0.109	0.050
Cxx 109	C	0.395	0.000	0.050
Cxx 172	I	0.167	0.067	0.000
Cxx 250	I	0.348	0.016	0.050
Cxx 123	I	0.146	0.000	0.000
Cxx 123	J	0.104	0.000	0.000

We determined a distribution of POI (Fig. 2.3A) and I_I scores (Fig. 2.3B) for the captive red wolves using allele frequencies from the Algonquin population representing the eastern Canadian wolf and from the Texas coyote population. The majority of captive red wolves overlapped with the distribution of the eastern Canadian wolf population for both assignment tests. If coyote genetic material resulted in the apparent similarity of these wolves, we would have expected the red wolf to fall within or closer to the distribution of its geographic neighbor, the Texas coyote population, and not the geographically distant population of Algonquin Park eastern Canadian wolves.

We further assessed the eastern Canadian wolves and captive red wolves in the context of the gray wolf using I_I indices and POI values using allele frequencies from the Algonquin Park eastern Canadian wolves and a gray wolf population from the Northwest Territories. The POI estimates indicated eastern Canadian wolves and red wolves clustered together and distinctly from both the gray wolves and the Texas coyotes (Fig. 2. 4A). The I_I indices from two comparisons (Algonquin wolves vs. Texas coyotes and Algonquin wolves vs. Northwest Territories) were plotted (Fig. 2.4B). The eastern Canadian wolves and red wolves clustered together and away from gray wolves in both I_I comparisons and the two wolves grouped closer to coyotes in the Algonquin/Northwest Territories comparison. The Algonquin wolves and red wolves clustering away from the distribution for gray wolves using both assignment tests suggested little or no gray wolf (*C. lupus*) genetic material in these populations. This finding was inconsistent with the eastern Canadian wolf representing a sub-species of the gray wolf, *C. lupus*, and

Figure 2.3. A comparison of measures of individual indicies for captive red wolves, canids from Algonquin Park and Texas, **A.** Log-likelihood individual indices (I_I) from captive red wolves (n=60) and canids from Algonquin Park (n=49) and Texas (n=22). The I_I were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Algonquin Park population and Texas coyote population, respectively. **B.** A plot of the log Probability of Identity (POI) values from captive red wolves (n=60) and wolves from Algonquin Park (n=49) and Texas (n=22) using the allele frequencies from the Algonquin Park population and Texas coyote population, respectively.

B.

Algonquin POI

Texas POI
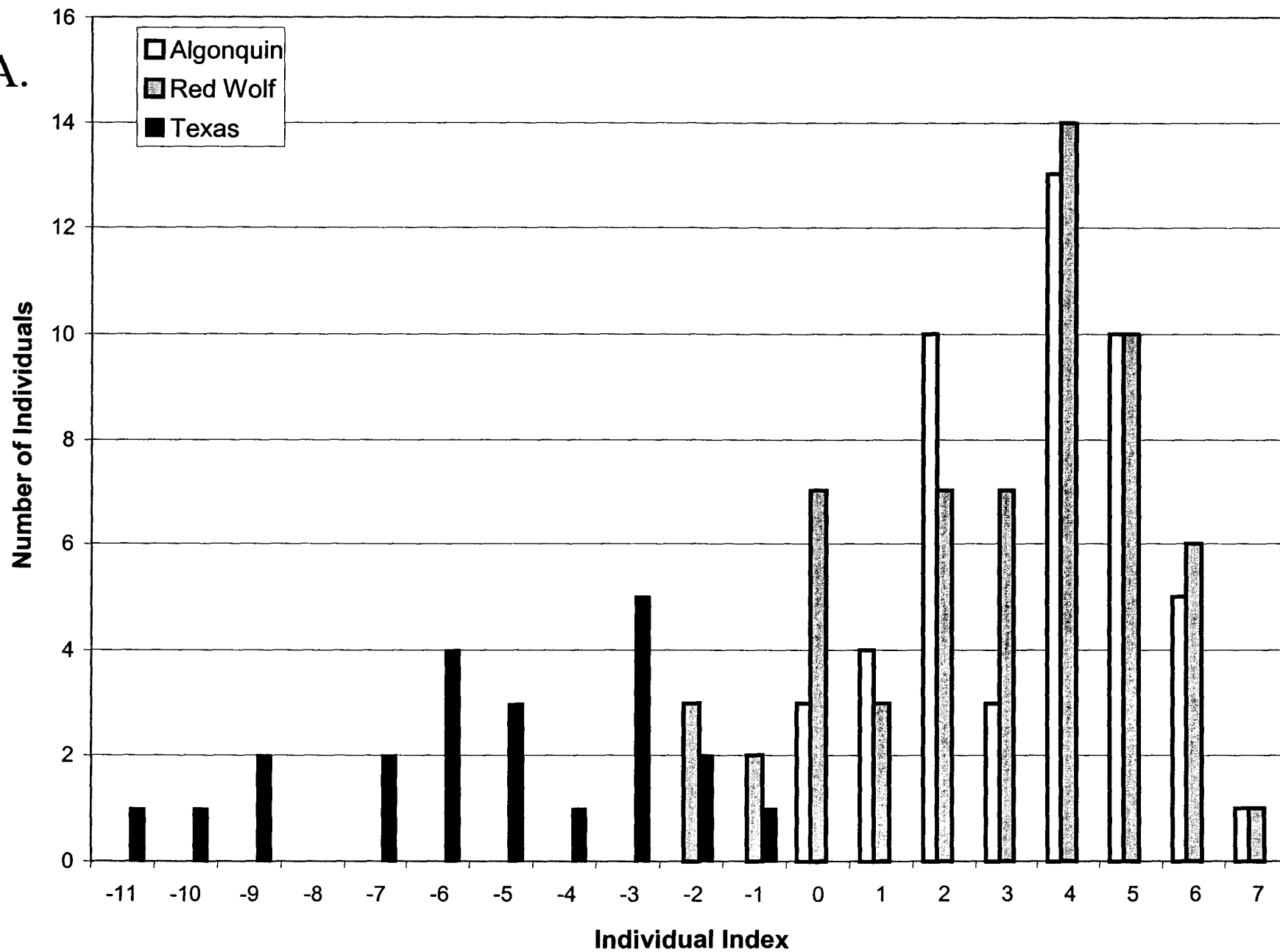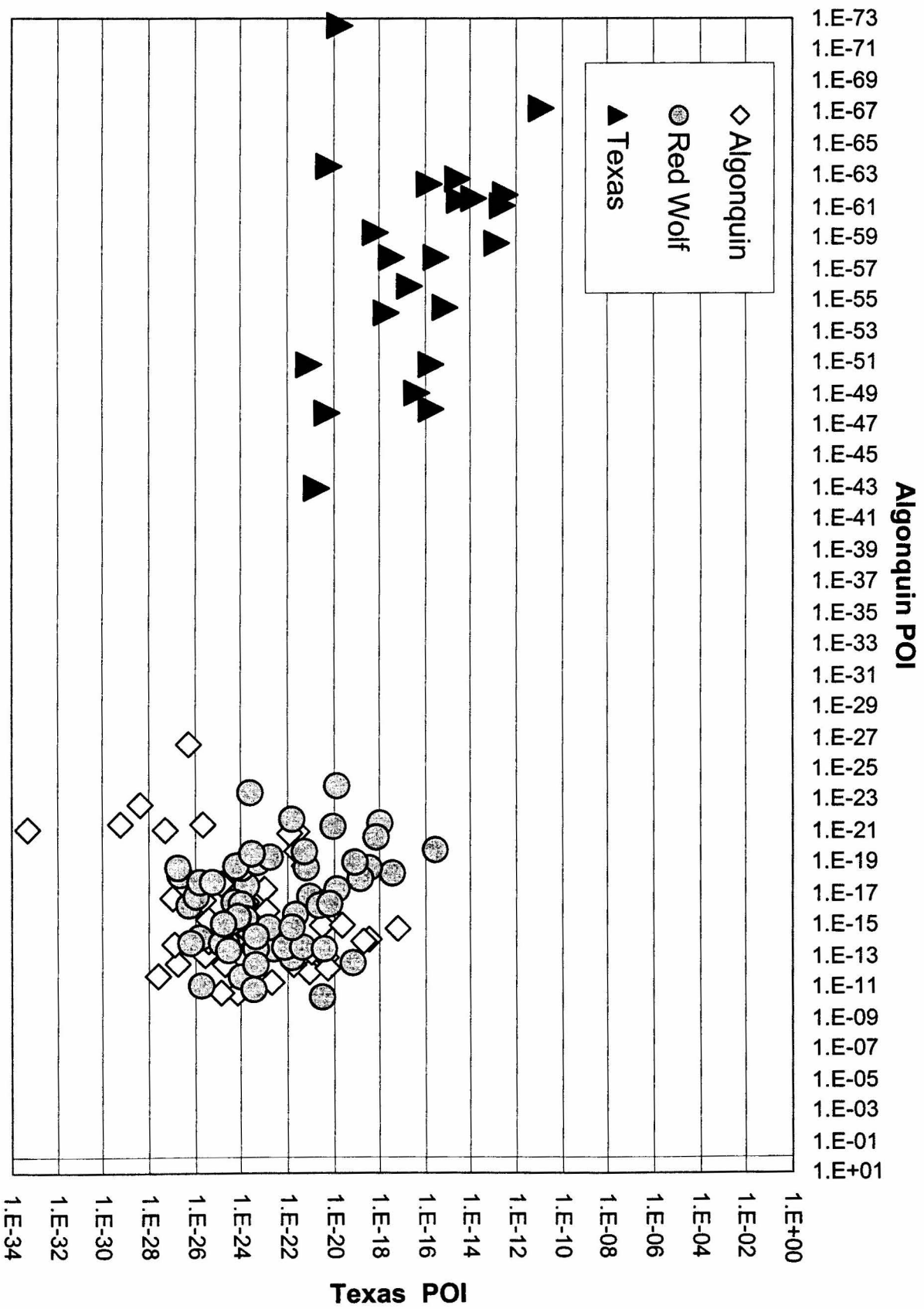
Figure 2.4. A comparison of measures of individual indicies for captive red wolves, canids from Algonquin Park, Northwest Territories and Texas, **A.** Plot of log-likelihood individual indices (I_I) from captive red wolves (n=60) and wolves from Algonquin Park (n=49), Northwest Territories (n=67) and Texas (n=20). The I_I were calculated for each individual animal DNA profile at 8 microsatellite loci using the allele frequencies from the Northwest Territories wolf population and Texas coyote population, respectively. **B.** A plot of the log of Probability of Identity (POI) values from captive red wolves (n=60) and wolves from Algonquin Park (n=49), Northwest Territories (n=67) and Texas (n=22) using the allele frequencies from the Algonquin Park population and Northwest Territories population, respectively.

A.
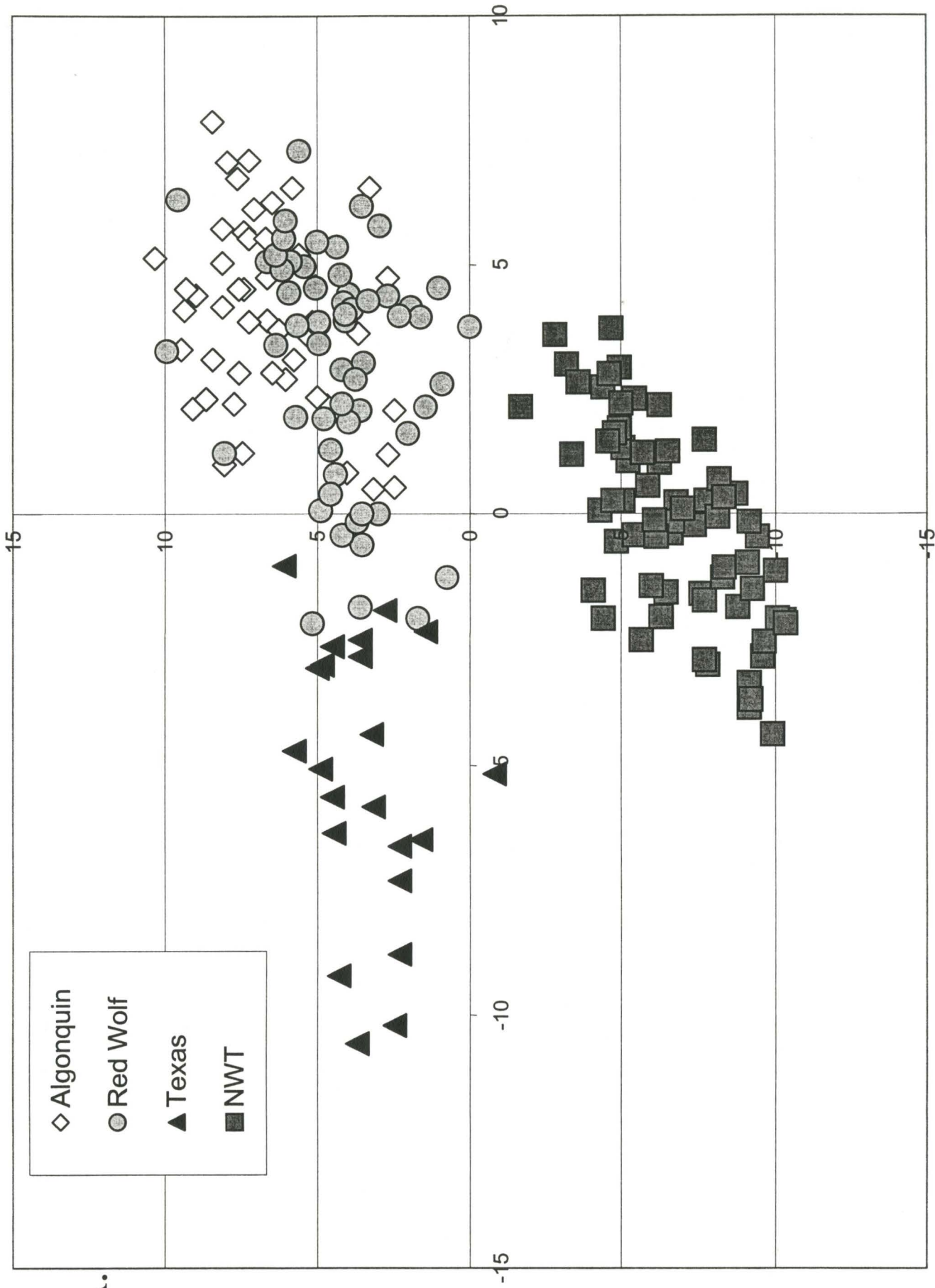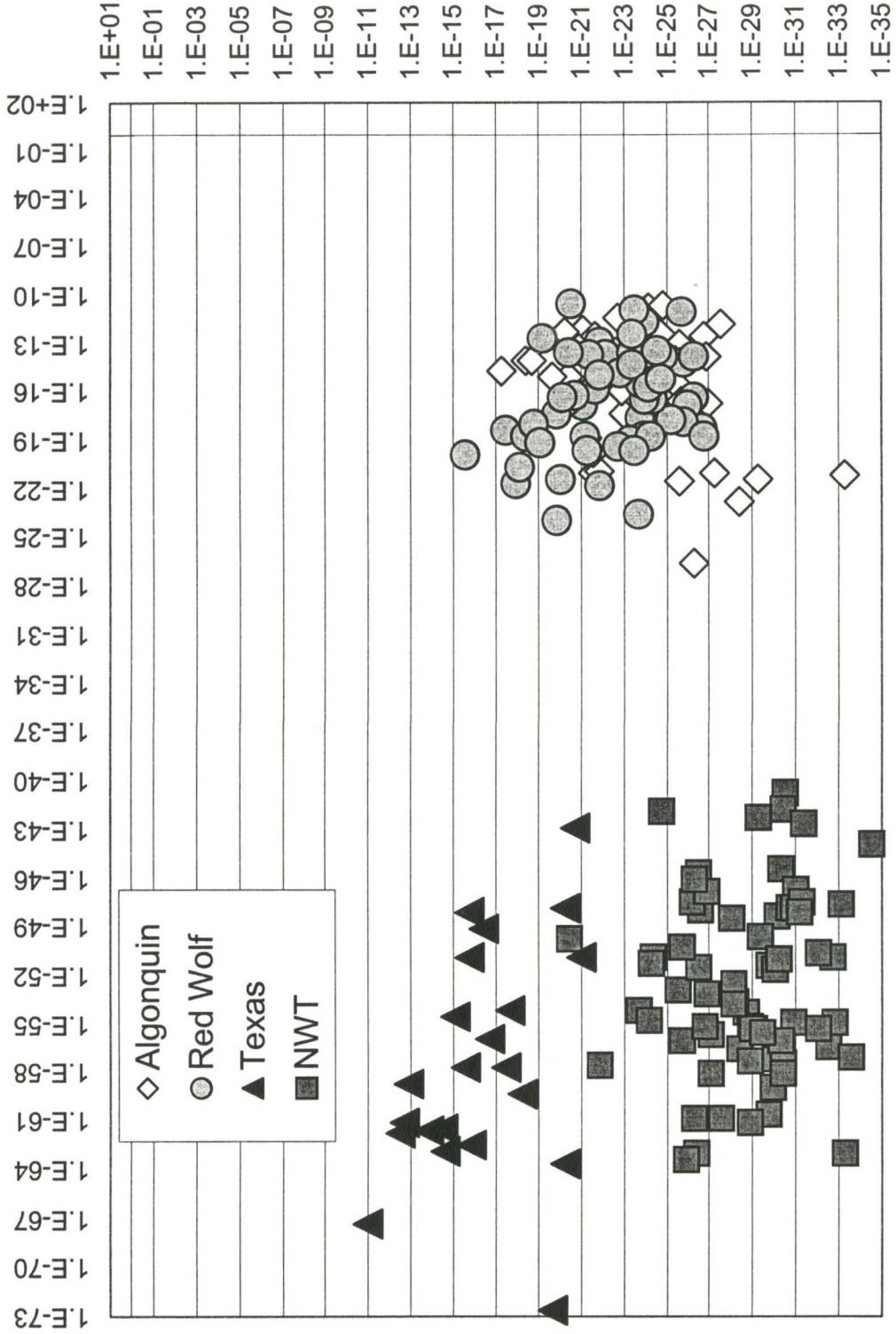
B.

inconsistent with the gray wolf having a significant contribution in the formation of the red wolf.

Given the apparent absence of gray wolf genetic material, we examined mitochondrial control region sequences from the captive red wolves, from teeth samples collected in Algonquin Park and elsewhere in Ontario during the 1960s and from Texas coyotes. Historic Ontario wolves had approximately 30 years of contact with coyotes and represent the best available natural sample set of the eastern Canadian wolf. We found no gray wolf control region sequences in any red wolf or any historic samples collected in Algonquin Park (n=19) consistent with the microsatellite assignment tests. However, we identified one haplotype (C1) in the park animals and surrounding area that was not found in coyotes and the sequences of which were divergent from those in coyote (Fig. 2. 5A). Among the red wolf samples, we identified a distinct haplotype (C2) not found in coyotes. A third haplotype (C3) was observed in a wolf from Manitoba that grouped with the historic eastern Canadian wolf haplotypes. Phylogenetic analyses grouped the eastern Canadian wolf and red wolf haplotypes (C1-C2) and C3 haplotypes away from the coyote haplotypes in a neighbor-joining analysis (Fig. 2.5B).

The historic Algonquin Park samples contained the C1 haplotype in 7 of 13 animals we were able to obtain control region sequences from 9 of 12 red wolves contained the C2 haplotype. The presence of the related C1 and C2 sequences in the geographically separated red wolves and eastern Canadian wolves but not the Texas coyotes, is consistent with a common origin of these two wolves. The remaining samples in this

Figure 2.5. A comparison of haplotype divergence using, **A.** Minimum-spanning tree

for 238 b.p. of control region haplotypes from red wolf, eastern Canadian wolf and

coyote. Sequences obtained from this study are labeled with a C designation, i.e. Canis-1

(C1). Gray shaded haplotypes indicate haplotypes found in eastern Canadian wolves

(lycaon) and striped haplotypes indicate red wolf (rufus) haplotypes. Dashes between

haplotypes indicate the number of base pair substitutions or insertion/deletions. **B.**

Neighbor-joining tree of sequence divergence for 238 b.p. of gray wolf, red wolf, eastern

Canadian wolf and coyote control region haplotypes. The lycaon/rufus lineage has two

nucleotides in the mtDNA control region common with *C. lupus* but different from *C.*

latrans which accounts for the proximity of *C. lupus* mtDNA to the lycaon/rufus

haplotypes. The scale represents 0.100 or 10.0% sequence divergence. Bootstrap values

are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data

sets. European wolf haplotypes (W1-W4) (Ellegren 1996) are provided. Sample

locations and corresponding haplotypes are as follows: red wolf captive breeding

program (C2, n=9, C19, n=3); Algonquin Park and surrounding areas (c. 1960's) (C1,

n=7; C9, n=1; C14, n=3; C17, n=1; C19, n=1); southern Ontario (c. 1960's) (C1, n=1;

C9, n=1; C14, n=2, C19, n=4); north of Algonquin Park (c. 1960's) (C1, n=1; C16, n=1,

C23, n=1); northern boreal region of Ontario (c. 1960's) (C23, n=1); northwestern

Ontario (c. 1960's) (C13, n=2; C24, n=1); Manitoba (C3, n=1; C22, n=1; C23, n=1);

Ohio (C5, n=1); Texas (C4, n=1, C6, n=2; C7, n=1; C8, n=1; C10, n=1; C11, n=1; C12,

n=1; C15, n=1; C18, n=2; C19, n=12; C20, n=2; C21, n=2); northern Quebec (C23, n=1),

NWT (C23, n=1), Fort Francis, Ontario (C23, n=1).

C16

C17

C15

C14

C18

C2

66

C1

C3

60

C24

99

C22

78

C23

C4

C5

C6

C7

C19

C20

C21

C10

C13

C11

C12

C8

C9
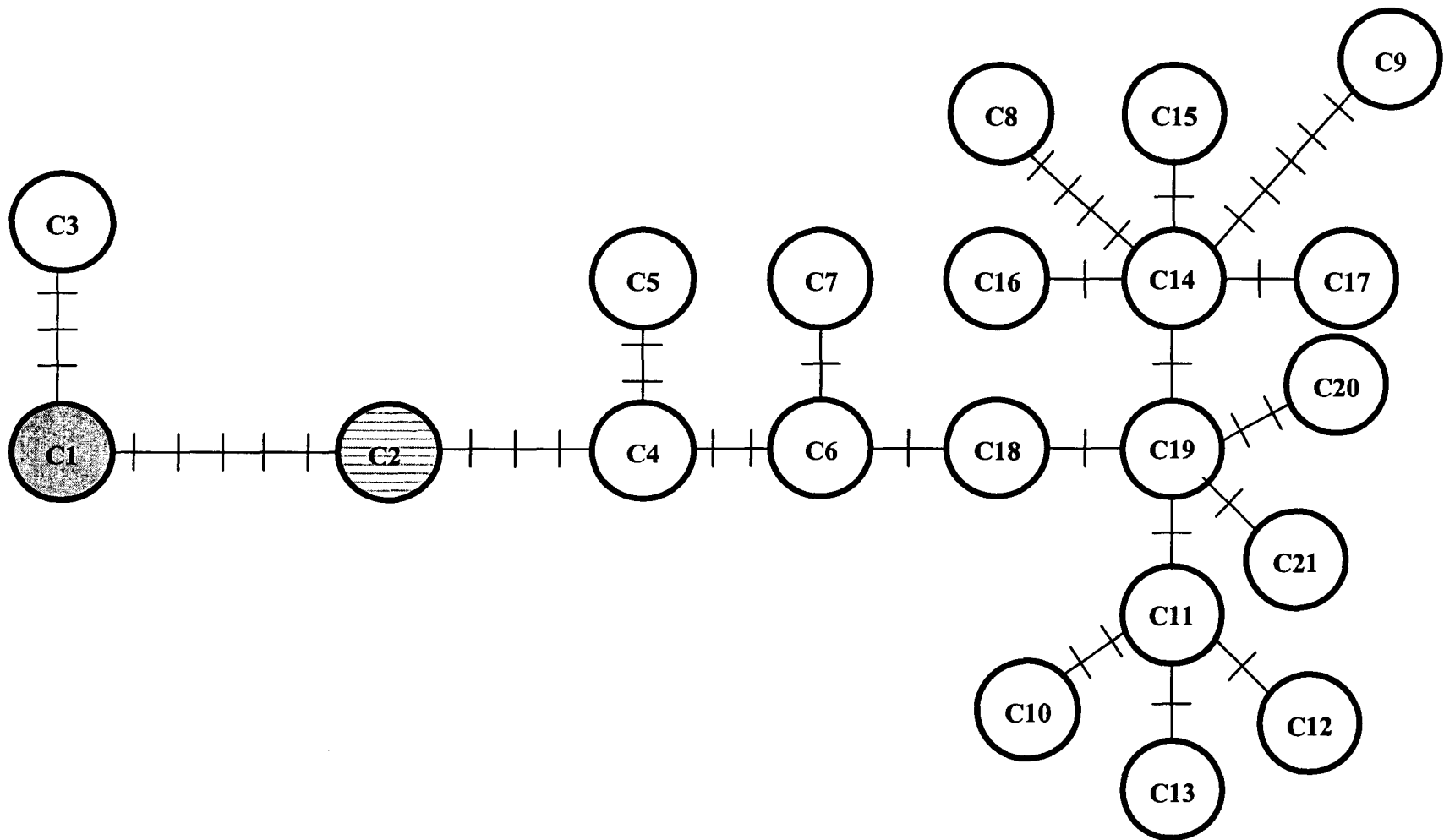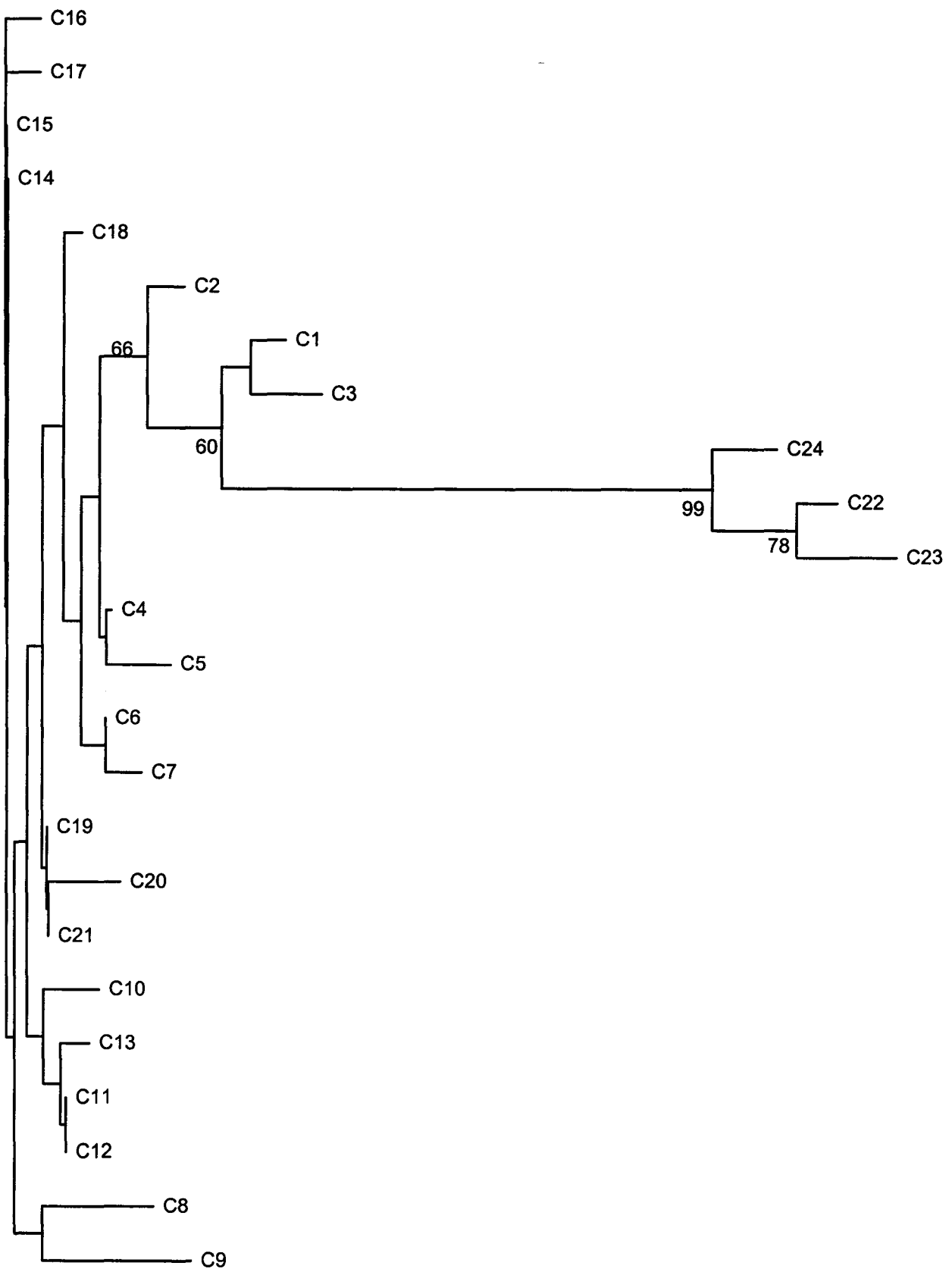
0.1

population contained coyote mitochondrial DNA sequences confirming that some level of hybridisation has also occurred.

The sequence divergence between the haplotypes observed in the eastern Canadian wolf and the red wolf haplotype was 2.1%. The intra-specific sequence divergence for coyotes (*C. latrans*) was 1.7 %. A comparison of the eastern Canadian wolf sequence (C1) to coyote sequences indicated 3.2 % sequence divergence and 2.3 % sequence divergence between the red wolf (C2) and coyote haplotypes. The sequence divergence of gray wolf (*C. lupus*) mtDNA from the haplotypes found in eastern Canadian wolves and red wolves was approximately 8.0%, and 10.0% between gray wolf and coyote haplotypes. The sequence difference observed between the eastern Canadian wolf sequences and the coyote sequences is consistent with 150,000-300,000 years separation, using a divergence rate of 1-2% per 100,000 years for the mammalian control region (Stewart and Baker 1994) and is consistent with the 1-2 million year divergence between gray wolves and coyotes (Kurten and Anderson 1980, Wayne 1993, Vila et al. 1997).

DISCUSSION

The similarity between the eastern Canadian wolf and the red wolf has been noted previously and both wolves were described as small eastern wolves long before the eastward expansion of coyotes (Brewster ad Fritts 1995). Neighbor-joining analysis of Nei's genetic distance using previously published data (Roy et al. 1994, Roy et al. 1996) and additional data we obtained from captive red wolf, other gray wolf and coyote populations again grouped the eastern Canadian wolf population and the captive red wolf

samples. One interpretation of this relationship was that "hybridizing wolves" of Algonquin, southern Quebec and Minnesota and the red wolf contained similar amounts of coyote genetic material (Roy et al. 1994). This interpretation did not seem consistent with genetic distances between the red wolf and the Texas coyotes, which was the closest coyote source population for the red wolf. The absence of common coyote alleles within Eastern Canadian wolves Park and the red wolf samples suggested that the close relationship observed between these two wolf populations was the result of a common wolf genetic origin. The application of assignment tests, an Individual Index (I_I) and Probability of Identity (POI), further supported the hypothesis that non-coyote derived parts of the genome were responsible for the similarity between the red wolf and the eastern Canadian wolf.

The presence of distinct control region haplotypes within the eastern Canadian wolves from the historic Algonquin Park population and the fact that captive red wolves clustered closer to coyotes than to gray wolves, supports the evolution of the eastern wolves independent of the gray wolf. These data indicate that, like the nuclear microsatellite DNA, the mtDNA of the eastern Canadian wolf/red wolf is not of gray wolf origin but similar to coyotes because of their relatively recent divergence from a common ancestor. It is unlikely that the eastern Canadian wolf mtDNA haplotypes obtained from the early 1960s represent the total introgression of coyote mtDNA as the Algonquin population would have had only 30 years of contact with the expanding coyote population and would require the replacement of gray wolf (*C. lupus*) mtDNA.

The coyote has been identified as the New World evolved canid species (Nowak 1979, Wayne 1993). Our data indicate a divergence in the North American canis mtDNA lineage of two types: 1) the red wolf and eastern Canadian wolf; and 2) coyote. We propose a model (Fig. 2.6) in which these two lineages diverged within the mid-Pleistocene, 150,000-300,000 years ago and came into contact in post-settlement time as a result of extensive habitat alteration. Further, the evolution of North American wolves and coyotes occurred independently of the gray wolf, *C. lupus*, that evolved in Eurasia 1-2 million years ago. We suggest the eastern North American wolf adapted to prey such as white-tailed deer within a forested habitat and the western coyote adapted to arid regions and smaller prey. The red wolf mtDNA haplotype, while showing sequence similarity to the eastern Canadian wolf mtDNA, is less divergent from coyote mtDNA and this may reflect continued contact with coyotes.

Several lines of evidence support a common origin for red wolves and eastern Canadian wolves: 1. The historic range of the eastern Canadian wolf overlaps with that of the present day red wolf and both would have existed in southern refugia during the Pleistocene (Nowak 1979, Brewster and Fritts 1995); 2. Pleistocene fossils suggest a small wolf inhabited eastern North America (Nowak 1995); 3. Species that evolved in the New World and diverged only 150,000-300,000 years ago are more likely to hybridize with each other than with the gray wolf. Lack of introgression of coyote DNA into western and even the Mexican gray wolf, *C. l. baileyi* (Roy et al. 1996, Lehman et al. 1991, Garcia-Moreno 1996) populations sympatric with coyotes suggests that eastern Canadian wolves and red wolves are the only wolves that hybridize readily with coyotes.

Figure 2.6. A model for the evolution of North American wolves. The progenitor to *C. lupus*, *C. lycaon* and *C. latrans* is indicated at the top. Divergence from this ancestor is generally accepted to have occurred 1-2 million years ago when the progenitor of *C. lupus* migrated to Eurasia. The North American species diverged 150,000-300,000 years ago into the eastern Canadian wolf /red wolf (*C. lycaon*) and the coyote (*C. latrans*). Recently, *C. lycaon* and *C. latrans* have come into contact and have subsequently hybridized. The Eurasian-evolved *C. lupus* returned to North America within the Pleistocene.

1-2 x 10^6 $_{YA}$

Ancestral Canid

1.5-3.0 x 10^5 $_{YA}$

Gray Wolf sub-species

**Eastern Canadian Wolf
& Red Wolf**
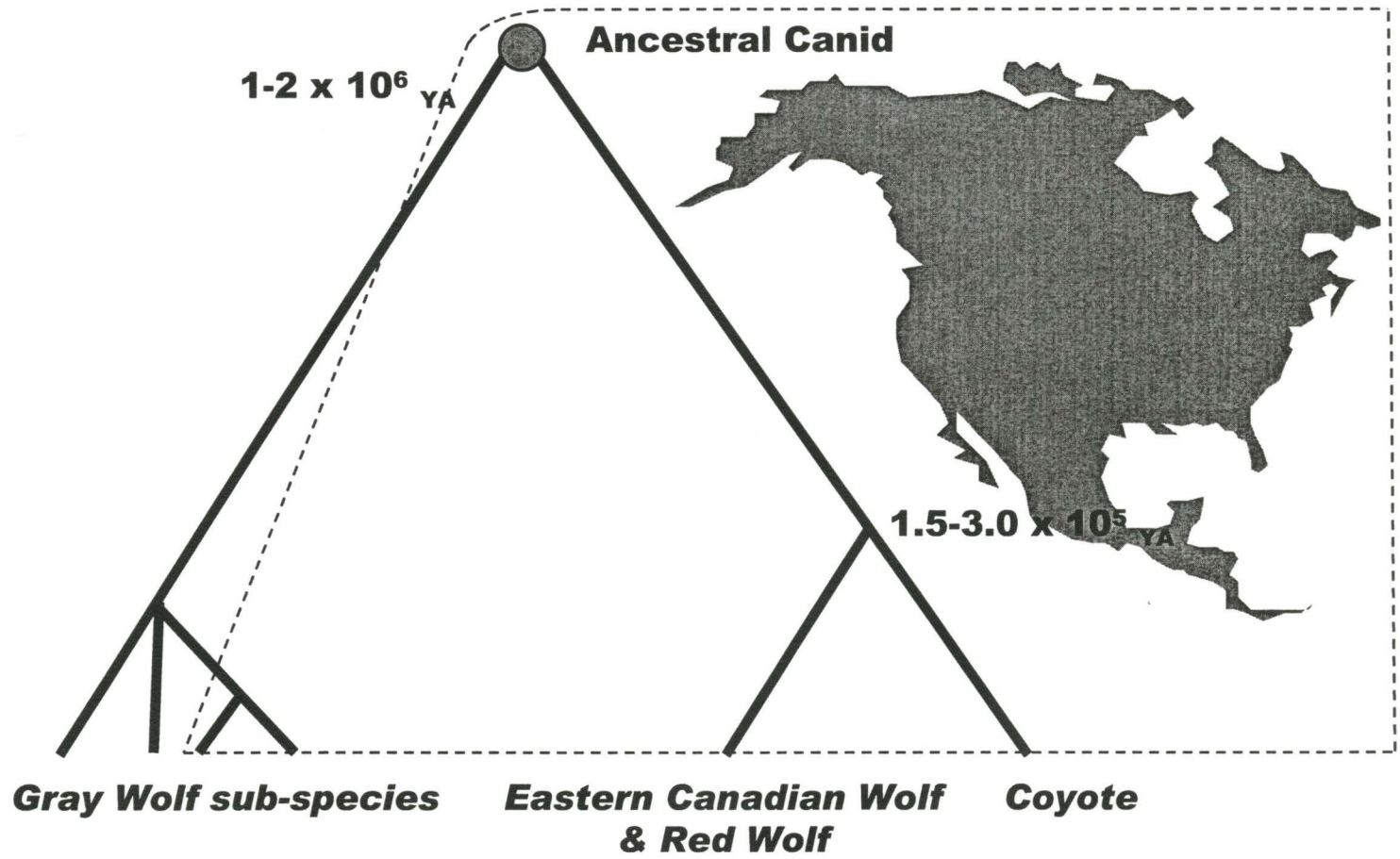
Coyote

The fact that the Mexican wolf shows no hybridisation with coyotes suggests that the smaller size of the eastern wolves is not reason for their hybridisation with coyotes.

The predisposition of the eastern North American wolves to hybridize with coyotes may represent an evolutionarily characteristic unique to these wolves, suggesting the red wolf (*C. rufus*) and the eastern Canadian wolf (*C. l. lycaon*) share a common origin. Several additional lines of evidence are consistent with the hypothesis of a common origin between these wolves. First, the historic range of *C. l. lycaon* overlaps with that of the present day *C. rufus*. Further, it has been proposed that these species existed in southern refugia during the Pleistocene (Nowak 1979, Brewster and Fritts 1995). Second, skull morphology comparisons indicate similarities between *C. rufus* and *C. l. lycaon* (Nowak 1979, Lawrence and Bossert 1967, Lawrence and Bossert 1975, Nowak 1995). Algonquin Park wolves have previously been described as a remnant red wolf population, classified as *C. niger* at the time (Stanfield 1970). A common origin also has been suggested by Mech (1971) who stated "if the red wolf is a hybrid between the wolf and coyote, it would be this sub-species (*C. l. lycaon*) of wolf that is involved".

The only evidence contrary to the hypothesis of a North American-evolved wolf is the apparent presence of gray wolf, *C. lupus*, mtDNA haplotypes within 6 red wolf samples collected from the southeastern U.S. and samples from the northwestern Great Lakes region. *Canis lupus* mtDNA haplotypes were identified in 3/6 (Wayne and Jenks 1991) and 3/11 pre-1940s (Roy et al. 1996) red wolves from the historical range of *C. rufus*. We question whether these six samples were red wolves, as the historic range of *C. rufus* has been identified as overlapping with the distribution of the gray wolf sub-species

C. l. nubilus (Schwartz and Schwartz 1991; Caire et al. 1989) and a Texas range of *C. l. baileyi* (Nowak et al. 1995). Gray wolf mtDNA was also found in 16% of the 77 animals previously analyzed (Wayne and Jenks 1991) from the region where they were selected for the breeding program. Strict morphological criteria were used to classify the animals as red wolf, coyote or red wolf/coyote hybrid and 44 were selected. Subsequent selection of the most representative red wolf types provided 17 animals were used as founders. We suggest the 12 animals with gray wolf mitochondrial DNA from the original 77 may have been of *C. l. nubilus*, *C. l. baylei* or *C. l. familiaris* origin.

Wayne et al. (1998) stated that "genetically, the historic and recent red wolves were extremely similar suggesting they were derived from a single gene pool" which implies these samples accurately represent red wolves. Nowak and Federoff (1998) expressed concerns about the focus on samples for genetic analyses collected from the historic south central range and not the eastern range of the red wolf. We agree this is a problem, but not for the same reason. Including samples from this region that may represent the sympatric or integradated forms that include gray wolf and hybrid samples within the "red wolf" samples. Although distinct morphological differences exist between the red wolf and the Plains wolf (*C. l. nubilus*), morphologic overlap exists between these two species (Nowak 1979, Lawrence and Bossert 1967, Lawrence and Bossert 1975, Nowak 1995) and pelage color is too variable for specific identification. Therefore, identification of individual specimens based solely on morphology is questionable and a rigorous assessment of samples should be applied in characterizing wolves.

The problem of sympatric ranges of wolf and coyote species also exists in the western Great Lakes region. Northwestern Ontario and Minnesota contain the ranges of eastern Canadian wolves, Plains wolves (*C. l. nubilus*) and coyotes (*C. latrans*). Although the current sub-species distribution of *C. lupus* does not include the eastern Canadian wolf in this region (Nowak 1995), other assessments did (Nowak 1979, Brewster and Fritts 1995) and the presence of a divergent eastern Canadian wolf mtDNA haplotype in Manitoba (C4) supports an extended western range. A number of wolves from the Great Lakes region may have been previously identified with a lycaon/rufus haplotype, although the resolution of the RFLP and cytochrome b markers (Roy et al. 1996, Lehman et al. 1991), would not have resolved it from other coyote haplotypes. Northwestern Ontario, Isle Royale, Minnesota and Manitoba animals contained coyote mtDNA haplotypes not found in extant coyote populations. The original interpretation was that several waves of coyotes expanded into this region, hybridized and then the local coyote population became extinct (Wayne and Lehman 1992); this seems inconsistent with a large panmictic North American coyote population (Roy et al. 1994). These haplotypes are potentially in the same group as the lycaon/rufus lineage. Similarly, a coyote–like haplotype, that was diagnostic to the red wolf breeding program and not coyotes, was found in 23/30 of the initial animals (Wayne and Jenks 1991).

In summary, much of the Nowak/Wayne debate surrounding the red wolf has focused on the presence of coyote genetic material in red wolves (Wayne et al. 1992, Wayne et al. 1998, Nowak 1992, Nowak and Federoff 1998). However, the main issue stems from the claim that gray wolf mtDNA occurs in red wolves and eastern Canadian

wolves. It is generally accepted that the gray wolf, *C. lupus*, evolved in Eurasia (Nowak 1978, Wayne 1993, Vila et al. 1997). Nowak has proposed that a coyote-like progenitor originating in North America diverged on two continents evolving independently into the red wolf and gray wolf. If a wolf evolved in North America then the mtDNA in this canid should be more similar to coyotes, *C. latrans*, than gray wolves, *C. lupus*, which was observed in historic eastern Canadian wolves and the captive red wolf program. Wayne's hypothesis is that gray wolves and coyotes hybridized to form the red wolf. The support for this hypothesis was the absence in red wolves of distinct genetic markers not found in coyotes or gray wolves. We have identified a group of mtDNA control region sequences more closely related to coyotes than gray wolves that are specific to the red wolf and the eastern Canadian wolf (Fig. 2.5). The mtDNA data support the microsatellite data that indicates a close relationship between the red wolf, *C. rufus*, and eastern Canadian wolf, *C. l. lycaon*. Furthermore, the absence of gray wolf mtDNA and the distribution of assignment test scores away from the gray wolf distribution in captive red wolves and Eastern Canadian wolves support the evolution of a small North American wolf independent of the gray wolf. The data presented leads to the formal rejection of the hypothesis that the red wolf and the eastern Canadian wolf are hybrids of coyotes and gray wolves. Furthermore, we also reject the hypothesis that the eastern Canadian wolf is a sub-species of the gray wolf. At present the red wolf exists as the species *C. rufus*, however, based on historical taxonomic classifications, the eastern North American wolves would require the classification *C. lycaon*.

Assuming the proposed taxonomic revision is accepted, our findings have broader biological, ecological and conservation implications. The present range of the North American-evolved eastern Canadian wolf likely includes northwestern Ontario, Minnesota and Manitoba. These areas may contain two different species of wolves, the eastern Canadian wolf and the gray wolf and it is presently unclear to what extent these two wolves might interbreed. What is now considered a single population of gray wolves may be two sympatric species or hybrid canids. We are presently examining the amount of inter-breeding between *C. lupus* and *C. lycaon*. Conservation of wolves in North America is dependent on an assessment of population sizes and this can only be made when the species are clearly identified.

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Preface

This chapter was written with co-authors and formatted for submission to a peer-reviewed journal and is therefore a self contained manuscript with an individual reference section.

Chapter 3: Characterization of wolves across Ontario using mitochondrial and microsatellite DNA profiles.

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Status: Submitted to the Conservation Biology for review.

Contribution: Autosomal microsatellite and mitochondrial DNA data collection done by S.K.G and A.D.G.. Mitochondrial data collection done by S.K.G. and I.L. Some data analysis by P.J.W. Writing and majority of data analysis done by S.K.G. Research was conducted under the supervision and guidance of B.N.W.

Chapter 3

Characterization of wolves across Ontario using mitochondrial and microsatellite DNA profiles.

ABSTRACT

Four "types" or "races" of wolves have been previously described in Ontario. (1) A subspecies of the gray wolf (*Canis lupus hudsonicus*) inhabiting the sub-arctic tundra. (2) A "race" ("Ontario type") of a second gray wolf subspecies, the eastern Canadian wolf (*C. l. lycaon*) that inhabits the boreal forests and much of the Hudson Bay Lowlands. (3) A second "race" ("Algonquin type") of C. *l. lycaon* that inhabit the deciduous forests of the upper Great Lakes. (4) A small wolf ("Tweed type") that has been proposed to be a hybrid between the "Algonquin type" wolf and the expanding population of western coyotes, *C. latrans*. Using mitochondrial control region sequences and 8 microsatellite loci, we developed DNA profiles for 269 wolves from across Ontario. Clustering analysis was used to assess the relationship of wolves irrespective of location and this defined four groups that are typified by those in Algonquin Provincial Park, Pukaskwa National Park, Frontenac Axis and animals north of Lake Superior. These groupings were supported by the R_{ST} statistic and Nei's genetic distance. Assignment tests indicated that the "Tweed wolves" in this region are hybrids between the western coyote and *C. lycaon* and are representative of the eastern coyote. In the northwestern and northeastern Ontario upper Great Lakes regions, wolves also appear to be primarily *C. lycaon* and low

population differentiation among these regions and Algonquin Park suggests a larger metapopulation. Pukaskwa National Park maintains a small population of wolves, which are genetically closer to the gray wolves of the Northwest Territories than the surrounding *C. lycaon*. We suggest these represent an isolated remnant population of the "Ontario type", which was a gray wolf, *C. lupus*. The *C. lycaon* in the upper Great Lakes region contain gray wolf mitochondria and represent products of historic and/or continuing hybridization between *C. lycaon* and *C. lupus*.

Introduction

Central Ontario is inhabited by a mixture of wolf "types" and the area has been described as containing "Canis soup". Some of this complexity has been attributed to wolf hybridization with the coyotes, *Canis latrans*, which began colonizing Ontario in the early 1900s (Kolenosky and Standfield 1975). Although a similar expansion of coyotes into northwestern North America occurred there has been no similar formation of "Canis soup" in areas inhabited by western wolves. In 1975, Kolenosky and Standfield recognized four "types" or "races" of wolves in Ontario (Fig. 3.1A). In the sub-arctic tundra along the coasts of James and Hudson Bay, the subspecies of gray wolf, *C. lupus hudsonicus,* was identified. In the boreal forest of the Hudson Bay lowlands they recognized a "race" of another subspecies of gray wolf, *C. l. lycaon* and referred to it as the "Ontario type". In the deciduous forests of the upper Great Lakes, they differentiated a race of the same gray wolf subspecies, the "Algonquin type". The fourth type termed the

Figure 3.1. Previous assessments of the distribution of wolf types in Ontario. A. Hall and

Kelson 1959. **B.** Stanfield and Kolenosky, 1975 showing the estimated northern limit of

the "Algonquin type" and southern limit of the "Ontario type" in central Ontario. The

larger circle represents Algonquin Provincial Park and the smaller circles are locations of

"Tweed" wolves. **C.** Nowak, 1995. Showing most of Ontario occupied by the gray wolf

sub-species *C. l. nubilus* that he also placed throughout the Plains States of the US.

N
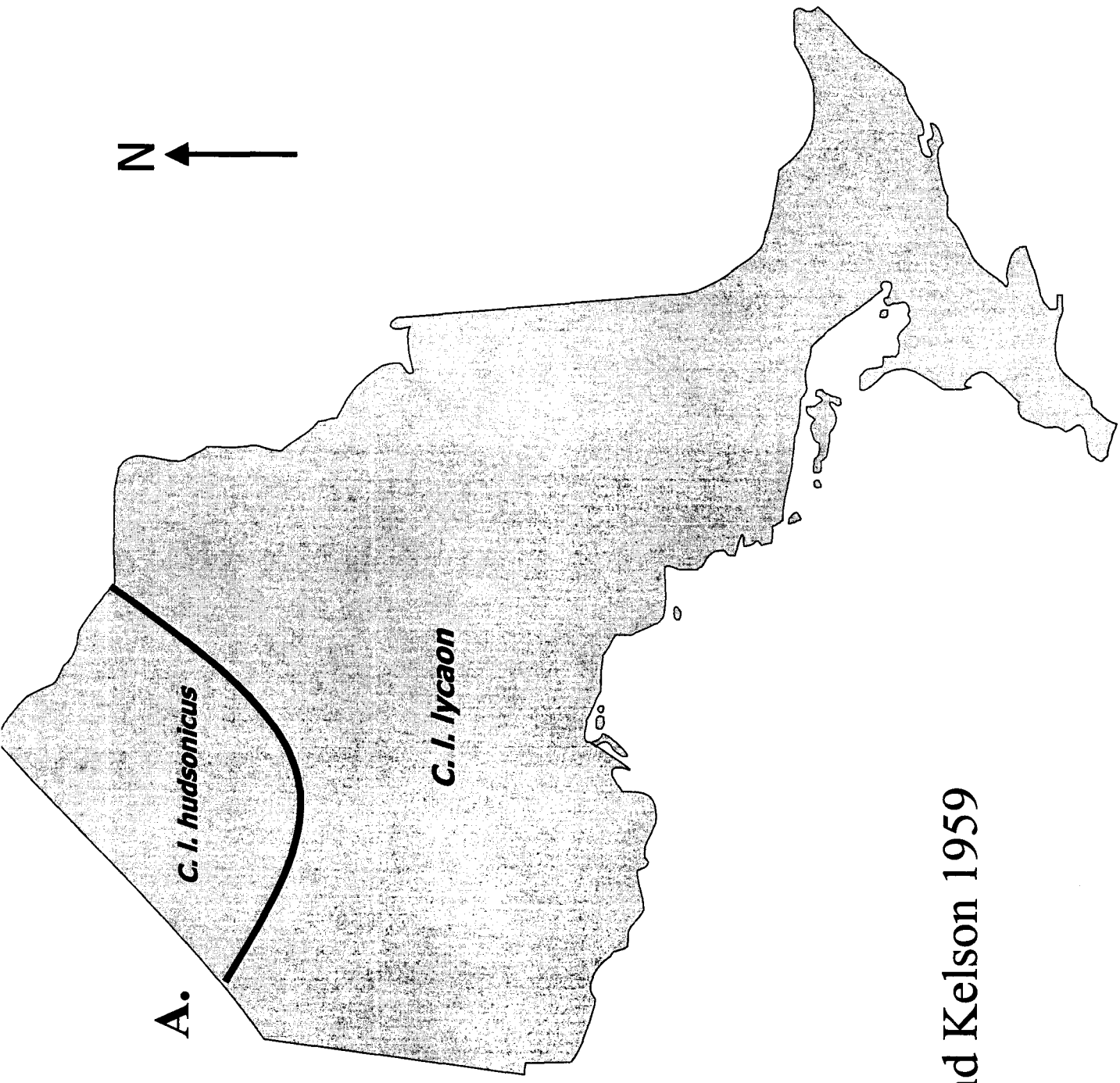
A.

C. l. hudsonicus

C. l. lycaon

Hall and Kelson 1959

B.

N ←

C. l. hudsonicus

Ontario Type
C. l. lycaon

Northwestern
Ontario

Northern limit Algonquin Type

Southern limit of Ontario Type

Algonquin Type
C. l. lycaon

Tweed
Type

Standfield and Kolenosky 1975

C.

C. l. occidentalis

C. l. nubilus

C. l. lycaon

N
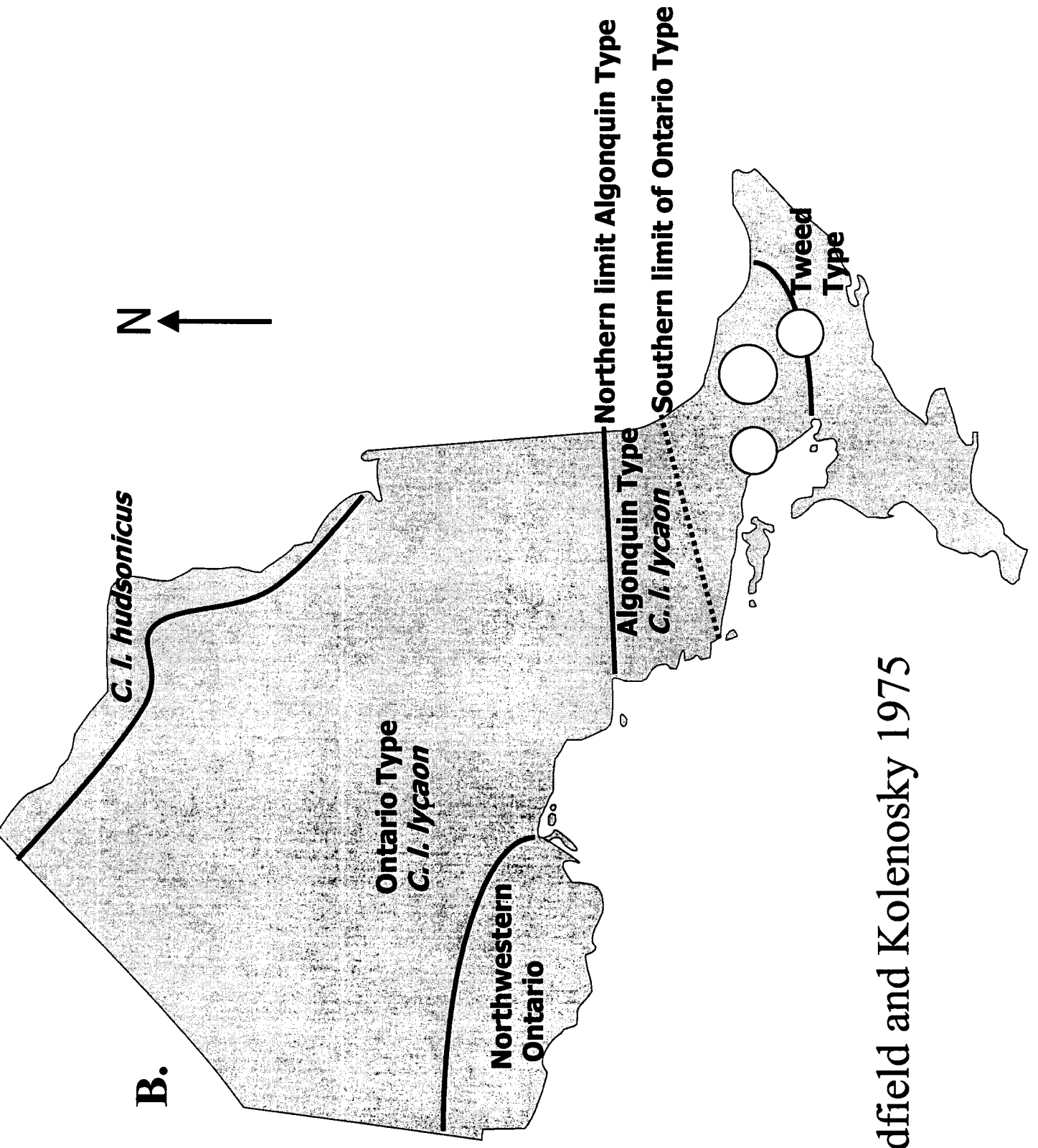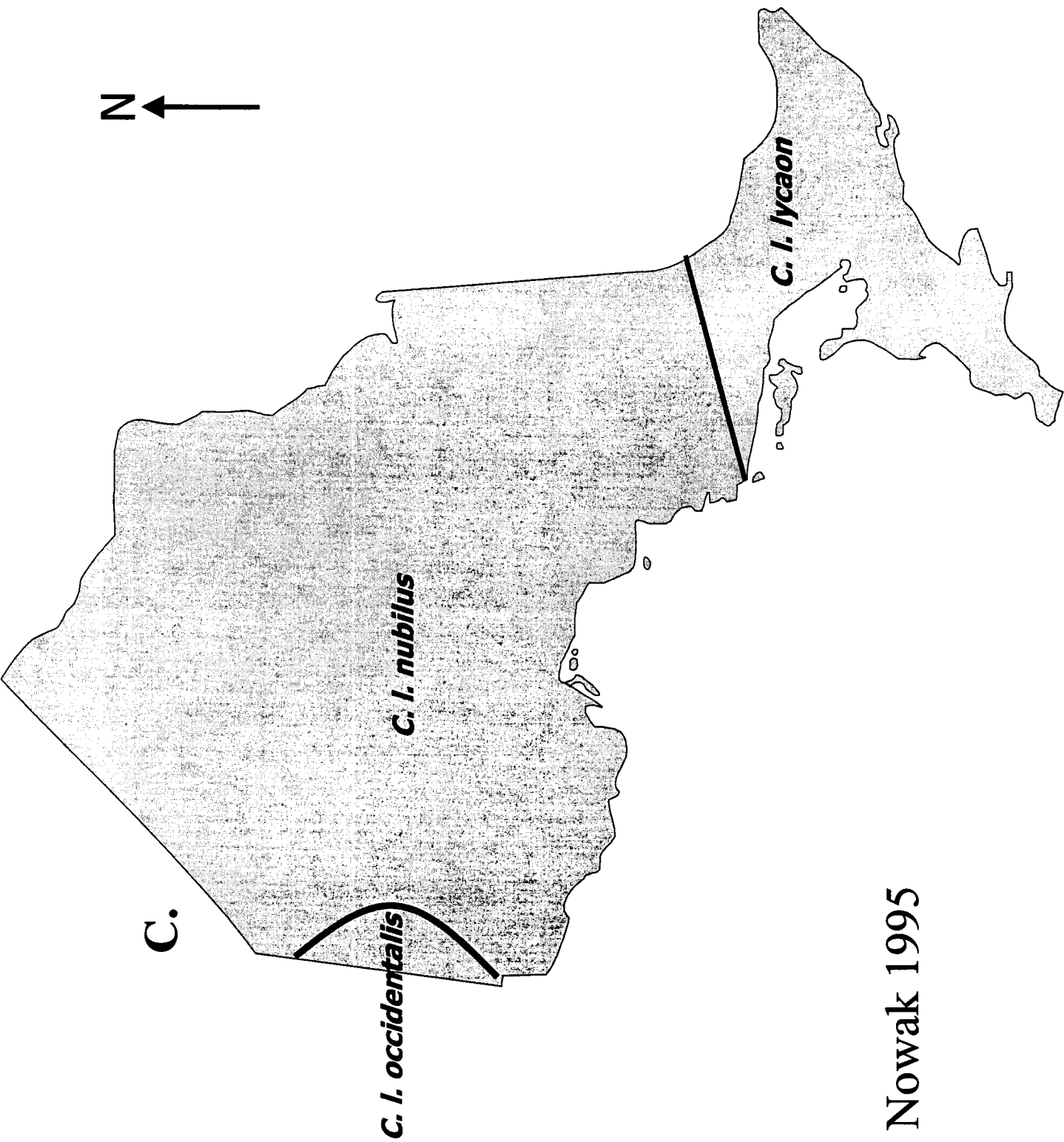
Nowak 1995

"Tweed wolf" appeared to have resulted from hybridization of the "Algonquin type", *C. l. lycaon,* with coyotes, *C. latrans.*

North American wolf taxonomy has undergone a series of revisions in the past century. The gray wolf, *C. lupus,* is thought to have originated in the Old World and migrated to the New World via the Bering Land bridge during the Illionian period of the Pleistocene glaciation, some 300,000 years ago (Nowak 1979, Kurten & Anderson 1980). The wide variation in color, size and weight in North American wolves was noted by many early authors and Miller (1912) attempted to provide a taxonomic framework to the morphological complexity. In eastern North America, he recognized 5 species including *C. lycaon* (eastern Canada) and *C. floridanus, C. lupus* var rufus and *C. frustror* that were later recognized as subspecies of the red wolf, *C. rufus.* Pockock (1935) recognized many of the species of Miller (1912) as subspecies of the gray wolf, *C. lupus,* but maintained the eastern Canadian wolf as *C. lycaon.* Following a number of revisions Young and Goldman (1944) produced a comprehensive treatment that considered the eastern timber wolf as a subspecies of the gray wolf (*C. l. lycaon*) and the only one present in Ontario. Hall and Kelson (1959) recognized *C. l. hudsonicus* along the coastal area of Hudson Bay in northern Ontario (Fig. 3.1B); however, Nowak (1983) and Mulders (1997) concluded *C. l. hudsonicus* should be reversed to *C. l. occidentalis.* Nowak (1983) further suggested grouping North American wolves into 5 subspecies based on Pleistocene refugia, with three of these occurring in Ontario based on similarities of skull measurements, *C. l. occidentalis, C. l. nubilis and C. l. lycaon*

(Nowak 1995) (Fig. 3.1C). Based on this proposed distribution most of Ontario was inhabited by *C. l. nubilis,* a subspecies originally assigned to the central Plains of the US.

There has been little consideration of the barriers to gene flow that originally must have been present to cause and maintain the differences among the "types" or subspecies. The Pleistocene ice sheets clearly had a major impact on the distribution of wolves and their ungulate prey in North America. Since the arrival of the Europeans, human impacts such as deforestation, farming, trapping and bounty hunting, extirpated wolves throughout most of the continent providing opportunities for the expansion of the coyote and the subsequent breakdown of the reproductive barriers between coyotes and eastern wolves. If the eastern wolves, *C. lycaon* and *C. rufus,* are North American-evolved wolves (Wilson et al. 2000, Nowak, 1983), a further level of reproductive isolation between them and the Eurasian-evolved gray wolf, *C. lupus,* would be expected. Kolenosky and Standfield (1975) described the northern limits of the "Algonquin type" wolves coinciding with the limits of white-tailed deer (*Odocoileus virginianus*) and deciduous forest. Their description of an absence of a cline between the "Ontario" and "Algonquin type" indicates recognition of a barrier to gene flow and is consistent with the theory that the boundary between the gray wolf and the eastern wolf was prevalent until the 1960s. There is some evidence from the Natural History of the Adirondacks that this frontier may have been south of the St Lawrence River in the mid 19th century. It describes the presence of two types of wolves similar (De Kay 1842) to the "Algonquin" and "Ontario" types of Kolenosky and Standfield (1975). The northward movement of the

"Algonquin" type in Ontario likely paralleled the northward movement of the white-tailed deer.

Earlier genetic studies (Lehman et al. 1991, Wayne et al. 1992; Wayne & Lehman 1992) of wolves in the Great Lakes region concluded that there are "hybridizing" wolf populations in northwest Ontario, Minnesota as well as in Algonquin Provincial Park and extending east in southern Quebec. This conclusion was based largely on the presence of both gray wolf and "coyote" mitochondrial DNA (mtDNA) in wolves in these areas. A recent study has proposed that the eastern Canadian wolf in Algonquin Provincial Park is closely related to the red wolf, *C. rufus*, and that both diverged from the coyote 300,000 years ago, while the gray wolf diverged more than one million years ago (Wilson et al. 2000). It was further suggested that this wolf should retain its original species designation of *C. lycaon* (Pockock 1935, Peterson 1966) rather than the presently accepted gray wolf subspecies designation of *C. l. lycaon*. The proposed evolutionary relationship of *C. rufus* and *C. lycaon* to the coyote, *C. latrans*, is consistent with the presence of a sister-species hybridizing in eastern North America and the absence of hybridization in western North America (Roy et al. 1994, Boyd & Forbes 1998).

In this study, we analyzed the control region of the mtDNA and eight microsatellite loci in 269 Ontario wolf samples. The primary objective of the study was to understand the genetic relationships of the four wolf "types" identified by Kolenosky and Standfield (1975) in the context of the coyote (*C. latrans*) and two distinct wolf species, the gray wolf (*C. lupus*) and the eastern Canadian wolf/red wolf (*C. lycaon*).

Materials and Methods

Sample Collection and DNA extraction

We analysed 269 samples from 6 geographic regions within Ontario (Figure 3.2): the Frontenac Axis (n=74); the Magnetawan Region (n=26); Algonquin Provincial Park (n=92); Northeastern Ontario, north of the French River and south of Highway 11 (n=33); Northwestern Ontario (n=30); Pukaskwa National Park (n=13) and one sample from Fort Severn on the coast of Hudson Bay (Table 3.1). DNA from blood and tissue samples was extracted by standard phenol-chloroform extraction methods described in Guglich et al. (1994). Included in this study are previously extracted samples from the Northwest Territories (n=66), Texas (n=26) and Ohio (n=22). These control samples represent gray wolves in the Northwest Territories unaffected by hybridization with coyotes and western coyotes from Texas and Ohio, which were present outside the range of the proposed distribution of the eastern timber wolf, *C. l. lycaon.*

Mitochondrial DNA Analysis

Sizing Assay for the Identification of New World (C. lycaon/C. latrans) and Old World (C. lupus) Control Region Sequences. A previously described method (Pilgrim et al. 1998) for distinguishing *C. lupus* mtDNA from *C. latrans* was modified to identify the presence or absence of gray wolf mtDNA within the 6 geographic regions. A 343-347 bp product of the mtDNA control region was amplified using primers described in Wilson et al. 2000. The control region was amplified in a total reaction volume of 10μl per tube using 25ng of genomic DNA, 200 μM dNTPs, 1x amplification buffer, 2 mM $MgCl_2$, primers 1 and 2 (0.2 μM) and 0.5 units of *Taq* polymerase (BRL). Products were

Figure 3.2. Map showing location of wolf samples from across Ontario. The samples (Table 3.1) were grouped into six regions in order to examine the types described by Kolenosky and Standfield, 1975. Northwest Ontario, northeast Ontario, Pukaskwa National Park, Algonquin Provincial Park, Magnetewan region to the west and north of Algonquin Provincial Park and the Frontenac Axis to the west and south of Algonquin Provincial Park. One animal came from Fort Severn (indicated by star) on the shore of the Hudson Bay.

Northern Ontario

Northwestern
Ontario

Pukaskwa National Park

Northeastern
Ontario

Magnetawan
Region

Algonquin Provincial Park

Frontenac Axis

Table 3.1. Wolf sample information including geographic location, number of samples, type of biological material and the source of the submitted material. Ontario samples and control samples from the Northwest Territories, Texas and Ohio are included. The one individual sampled from Fort Severn is included in the northeastern sample set.

Area[1]	Number	type	Source
Frontenac Axis	74	muscle	University of Waterloo[2]
Algonquin Provincial Park (1960s)	19	teeth	OMNR
Algonquin Provincial Park (1990s)	92	muscle and blood	University of Waterloo[2]
Magnetawan Region	26	muscle	University of Waterloo[2]
Northeastern Ontario (1960s)	46	teeth	OMNR
Northeastern Ontario (1990s)	34*	hide	North Bay Fur House
Pukaskwa National Park	13	blood	Parks Canada[3]
Northwestern Ontario (1960s)	11	teeth	OMNR
Northwestern Ontario (1990s)	30	hide	Laurentian University[4]
Total	345		

[1] Location of area shown in Figure 2.
[2] University of Waterloo samples – see Theberge et al. 1999 and Sears et al. 1999
[3] provided by Dr P. Paquet and F. Burrows.
[4] Laurentian University samples provided by Dr F. Mallory.
*Ft. Severn animal included in Northeastern Ontario sample set. Only used in assignment analysis.

amplified under the following conditions: 94°C for 5 minutes, 55°C for 30 seconds, 72°C for 30 seconds (1 cycle); 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds (35 cycles); 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes (1 cycle). Products were then mixed with 0.4 volume of formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size markers. The bands were visualized by autoradiography.

Microsatellite Analysis

Eight microsatellite loci (Ostrander et al. 1993, Roy et al. 1994, 1996) were amplified in a total reaction volume of 10μl using 25ng of genomic DNA, 200 μM dNTPs, 1x amplification buffer, 2 mM $MgCl_2$, unlabeled primers R and F (0.2 μM and 0.18 μM), radioactively labeled $\gamma^{33}P$-dATP (ICN) F primer (0.02 μM), 1 μg of Bovine Serum Albumin (BSA) (Pharmacia) and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 minutes, 55-65°C for 30 seconds, 72°C for 15 seconds (1 cycle); 94°C for 15 seconds, 55-65°C for 30 seconds and 72°C for 15 seconds (30 cycles); 94°C for 15 seconds, 55-65°C for 30 seconds and 72°C for 2 minutes (1 cycle). Products were then mixed with 0.4 volume of the formamide loading buffer and were heated at 95°C for 5 minutes before loading onto a 6% sequencing gel containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run adjacent to the samples to produce size markers for the microsatellite alleles. The bands were visualized by autoradiography.

Genetic Analysis

We analyzed allele frequencies at 8 loci for all 269 individuals representative of the different geographic regions of Ontario plus those individuals from the Northwest Territories, Texas and Ohio. Microsatellite loci genotypes were assigned based on the allele sizes of Roy *et al.* (1996). Descriptive statistics including allelic diversity, observed and unbiased heterozygosity (Nei 1987) and F_{IS} (Weir and Cockerham 1984) were generated using the program GENETIX (Belkir et al. 1999). Hardy-Weinberg Equilibrium (HWE) for each population-locus combination was tested for each region using the Markov Chain method in GENEPOP 3.1 through 1000 iterations (Guo and Thompson 1992).

We applied a Factorial Correspondence Analysis (FCA) on individual genotypes from Ontario in the program GENETIX (Belkir et al. 1999). FCA has been applied to examining the genotypic distributions of individuals, using the multilocus profile in a multivariate analysis without any *a priori* classification of individuals to geography or populations (She 1987, Roques et al. 2001).

We further assessed the number of genetic clusters or subpopulations (K) with no *a priori* assignment to a population based on geography using the computer program STRUCTURE (Pritchard et al. 2000). This program identifies genetically similar multilocus genotypes for individuals without any known population affiliation and provides a statistical assessment of the number, based on a likelihood measure, of the number of genetic clusters (K). We applied 1,000,000 iterations with a 30,000 burn-in period to determine the likelihood of the number K within the dataset (Pritchard et al.

2000, Rosenberg et al. 2001). An individual was assigned to a cluster if the proportion of the individual's genetic ancestry was assigned to a cluster greater than or equal to 75% of the iterations (Rosenberg et al. 2001). Individuals with 50-75% ancestry to a specific cluster were determined to have mixed ancestry with some contribution from that cluster. Individuals with less than 50% assignment to any cluster was classified as having unknown ancestry.

Nei's unbiased genetic distance (Nei 1978) was calculated using the programs and the chord genetic distance (Cavalli-Sforza and Edwards 1967) was calculated in the program GENETIX (Belkir et al. 1999). The likelihood-ratio distance (D_{LR}) using a probability-of-identity (POI) estimate for individuals from a specific geography was also generated for each region sampled (Paetkau et al. 1997). Neighbour-joining trees for each genetic distance was generated using the program NEIGHBOR in the computer package PHYLIP (Felsenstein 1993). The Northwest Territories gray wolves and western coyote control samples were used in the comparison. Bootstraps of the data were generated in PHYLIP using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE.

Population genetic structure was estimated using R_{ST} using the software program ARLEQUIN (Schneider et al. 2000). Significance levels were tested using 1000 permutations and a sequential Bonferroni correction for multiple pair-wise comparisons. R_{ST} is applied to loci undergoing a stepwise mutation process permitting homoplasy where two alleles of the same size can occur independently in two populations. This is best applied to this data set as two lineages are being assessed, *C. lupus* (Old World) and *C. lycaon/C. latrans* (New World), which diverged 1-2 million years ago.

To assess the relationship of an animal to one of two species an individual index was calculated over all eight loci using the following equation: I_I (individual index) $= \Sigma$ log (Xp_A/Xp_B) where p_A and p_B are the allele frequencies of allele X species A and B respectively. The log of the frequency of allele X in population A is taken over the frequency of the same allele X in the species. In the event an allele was absent from one species, the frequency of 1 allele in the population, i,e, sample size, was used. Once the log of all the ratios for every allele over all loci are calculated, the sum of the log values is calculated to give a LOD score. This LOD score assesses the origin of the alleles in each animal based on a ratio of frequencies from two species. If the populations have similar allele frequencies then the I_I values of individuals from both populations would follow a distribution around zero. An increasing positive score indicates an individual originated from species A and an increasing negative score indicates an individual originated from species B. A two dimensional plot was constructed using the program STATISTICA and 95% ellipses were calculated.

Results

Initially, the genetic characteristics of the wolves were assessed based on the geographic regions from which they were sampled (Table 3.1). The animals in the Frontenac Axis and Magnetawan regions contained only New World mitochondria (Table 3.2). Only 4/92 animals from Algonquin Provincial Park contained Old World mitochondria, which is not consistent with them being a subspecies of gray wolf. Most of the animals in Pukaskwa National Park contained Old World mitochondria, consistent

Table 3.2. Distribution of New World and Old World mitochondrial DNA control region in Ontario. This table lists the distribution of mitochondrial DNA across the six geographic populations in Ontario. The total number coincides with the number of individuals used in this study. The star represents the one individual from Fort Severn, which for this table was included in the northeastern Ontario group.

Population	Frontenac Axis	Magnetawan Region	Algonquin Provincial Park		Northeastern Ontario		Northwestern Ontario		Pukaskwa National Park
	1990	1990	1960	1990	1960	1990	1960	1990	1990
Old World	0	0	0	4	22	18	1	9	11
New World	74	26	19	88	24	14	10	21	2
Total	n=74	n=26	n=19	n=92	n=46	n=33	n-11	n=30	n=13

with them being gray wolves. There was a mixture of animals with Old and New World

mitochondria from Northwestern and Northeastern Ontario. In order to assess the effects

of isolation especially in the parks allelic diversity and heterozygosity values were

calculated for each of the regions (Table 3.3) and these were compared to Texas and Ohio

western coyotes and NWT gray wolves. The number of alleles present per locus was 5.0

in Pukaskwa and 5.4 in the gray wolves of NWT. There was an average of 7.6 alleles in

the Frontenac Axis "Tweed" wolves and 8.5 in the western coyotes. Expected

heterozygosity values were similar among the various populations. At locus cxx.200

deviations from Hardy-Weinberg Equilibrium (HWE) were observed in all groups except

for Algonquin, northeastern Ontario and Pukaskwa. In addition, locus cxx.377 deviated

from HWE in the gray wolves of Northwest Territories (Table 3.3).

We then assessed the genetic relationships of the animals sampled without

consideration of geography or population affiliation. Using the 269 Ontario profiles

generated from the 8 microsatellite loci, groupings were developed by the Factorial

Component Analysis (FCA) in the program GENETIX 3.3. In general, the genetic

distribution of the individuals formed three groups representing Frontenac Axis "Tweed

wolves", Algonquin Park eastern timber wolves and Pukaskwa gray wolves (Figure 3.3).

The northeastern and northwestern Ontario animals were spread between the Algonquin

Park wolf group, and the Pukaskwa Park group (Figure 3.3).

Similar groupings observed with FCA were found (Table 3.4) using the program

STRUCTURE. Most of the Frontenac Axis animals grouped into cluster 1 and most of

Algonquin Park animals fell within cluster 2. The Magnetawan samples were divided

Table 3.3. Information on geographic region, sample size (N), number of alleles, observed heterozygosity (Ho) and expected heterozygosity (H_E), F_{IS} and the specific loci deviating from Hardy-Weinberg Equilibrium (HWE) (P<0.050) are provided for Canis samples at 8 microsatellite loci. Calculations are listed for the six Ontario geographic regions and two control populations of NWT gray wolves and western coyotes using the method of Guo and Thompson (1992). Deviations from HWE followed applications of a Bonferroni correction for the number of loci (α = 0.050/8 = 0.006) are provided.

Geography	N	Alleles	H_O	H_E	F_{IS}	HWE	HWE$_{Corrected}$
Algonquin	49	6.6	0.650	0.650	0.001	2	0
						cxx.250,200	
Frontenac	74	7.6	0.684	0.712	0.039	2	1
						cxx.123,200	cxx.200
Magnetewan	26	6.5	0.677	0.702	0.036	1	1
						cxx.200	cxx.200
Northeastern Ontario	34	7.1	0.721	0.735	0.020	2	0
						cxx.225,250	
Pukaskwa	13	5.0	0.713	0.733	0.028	0	0
Northwestern Ontario	30	6.8	0.702	0.700	-0.003	1	1
						cxx.200	cxx.200
Western Coyotes[#]	61	8.5	0.681	0.741	0.081*	1	1
						cxx.200	cxx.200
NWT Gray Wolves	67	5.4	0.717	0.697	-0.029*	2	2
						cxx.377,200	cxx.377,200

\# Western coyotes include Texas and Ohio populations.
* Indicates F_{IS} values significantly different from 0.000.

Figure 3.3. Distribution of 269 individuals from the 6 geographic regions in Ontario using the FCA (Factorial Component Analysis). The *a priori* clustering analysis is based irrespective of designated geographic locations. Four clusters are identified; "Tweed" animals (Blue), Algonquin Provincial Park animals (Yellow), animals north of Lake Superior (White) and gray wolves represented by animals from Pukaskwa National Park (Gray).
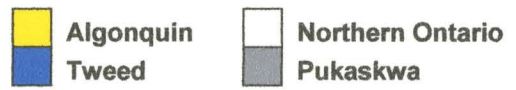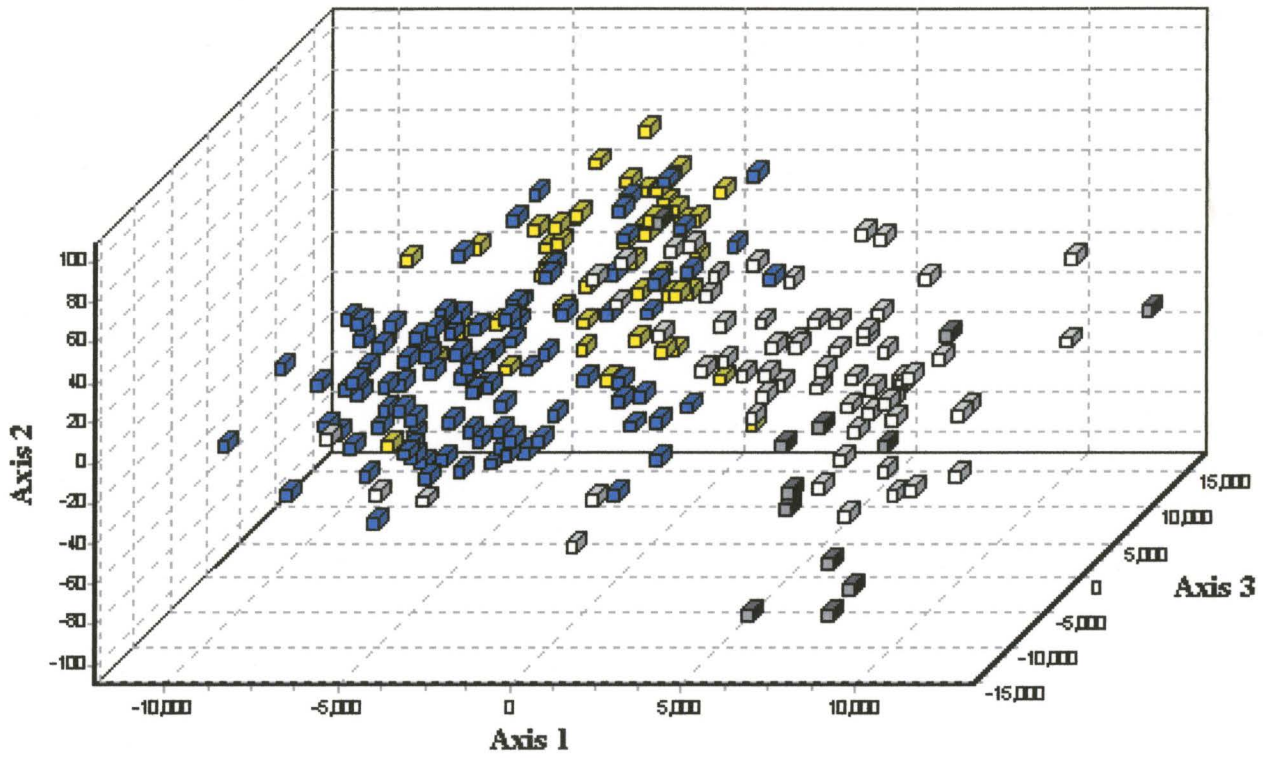
Table 3.4. The proportion of individual *Canis* samples assigned into 4 clusters inferred using STRUCTURE cluster analysis with no *a priori* population affiliation. Individuals were assigned when the proportion of the genome in an individual's ancestry clustered greater than or equal to 75% of the assignments. Values within the range of 50.0-75.0% were classified as individuals with detectable ancestry from a specific cluster. Values below 40.0% were classified as unknown ancestry.

Population	N	Cluster 1	Cluster 2	Cluster 3	Cluster 4	50-75%	Unknown
Algonquin	49	4	35	2	0	4	3
Frontenac	74	54	9	0	0	9	1
Magnetewan	26	9	4	1	1	9	2
Northeastern	34	3	2	12	3	13	1
Pukaskwa	13	0	1 (NW)	1	11 (1 NW)	0	0
Northwestern	30	1	3	7	6	10	3

amongst all clusters with the majority in 2. Cluster 1 represents "Tweed wolves" and cluster 2 represents southern eastern timber wolves that have been impacted by coyotes. Cluster 3 contains a mixture of animals with New World and Old World mitochondria and includes many of the animals from northeastern and northwestern Ontario and appears to represent eastern timber wolves impacted by gray wolves. Cluster 4 is representative of Pukaskwa gray wolves and includes the one sample from the coast of Hudson Bay.

The clusters represent a cline of individuals from eastern coyotes or "Tweed wolves" in the Frontenac Axis in the south, through the eastern timber wolves impacted by coyotes in Algonquin Provincial Park and eastern timber wolves impacted by gray wolves in northeastern and western Ontario to the gray wolves in Pukaskwa National Park. Unfortunately there is only one extreme northern sample, but it clusters with the Pukaskwa animals suggesting gray wolves occupy the large boreal region of Ontario. In order to further assess the relationships of the animals in the six geographic regions we estimated Nei's unbiased genetic distance using the microsatellite loci allele frequencies (Figure 3.4). Western coyotes from Ohio and Texas as well as gray wolves from NWT were included to provide a species basis for the comparison. The neighbour joining tree supports the cluster analyses with more coyote-like animals to the south, the eastern timber wolves in the middle and the gray wolves to the north. Animals from northeastern and northwestern Ontario are positioned between Algonquin and Frontenac Axis animals and gray wolves. The grouping of Algonquin Park animals with those in the Frontenac

Figure 3.4. Neighbour-joining tree based on Nei (1978) unbiased genetic distance. Bootstrap values are indicated at the nodes. The Northwest Territories dataset was used as an outgroup.

Western
Coyote

Algonquin

Magnetewan

63.5

Frontenac

86.1

Northwestern Ontario

Northeastern Ontario

70.8

Pukaskwa National Park

0.1

NWT
Gray Wolves
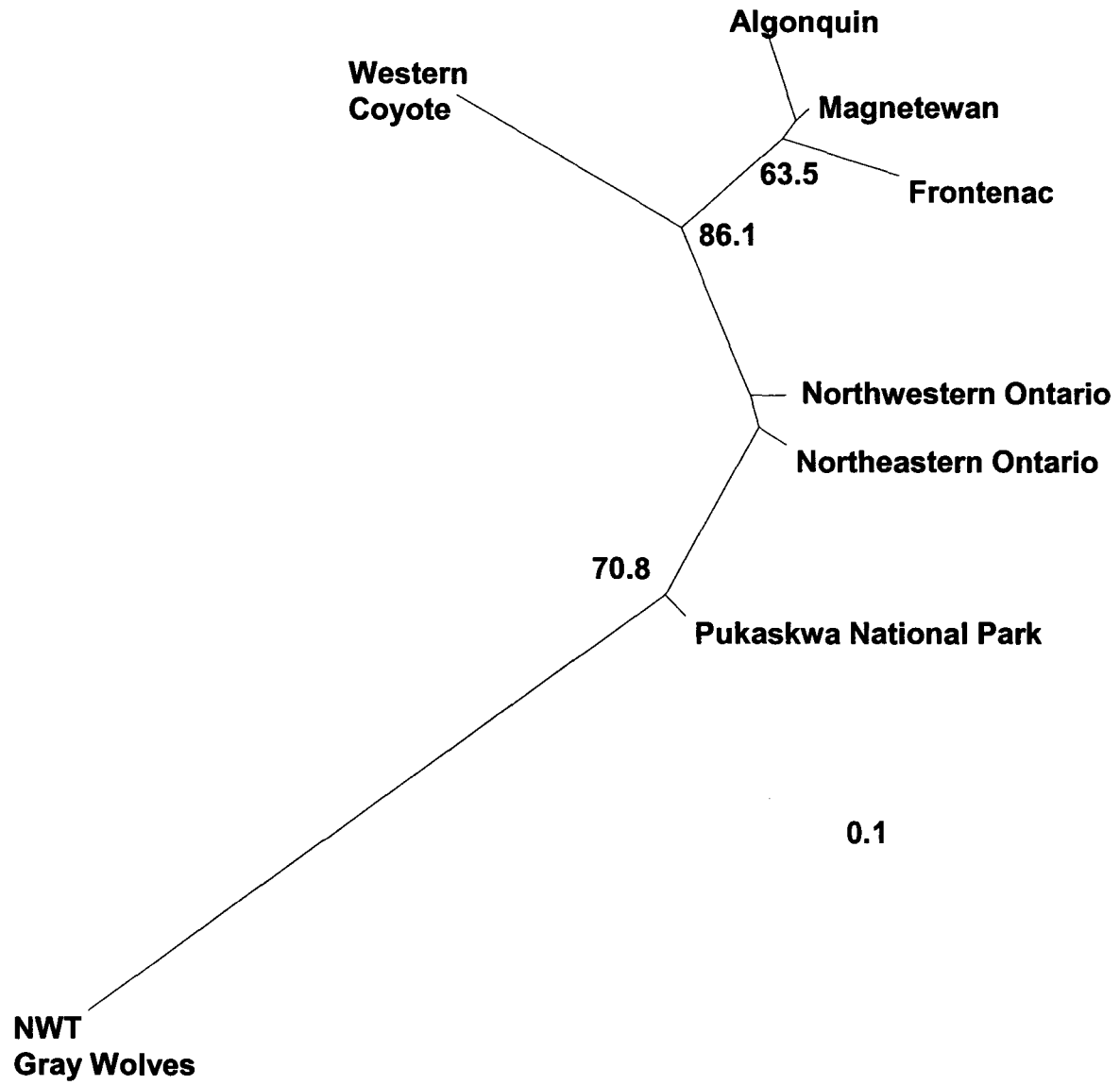
Axis and Magnetawan regions is consistent with these animals containing some level of western coyote genetic material.

To obtain information on connectivity and gene flow among the groups we assessed population structuring using R_{ST} (Slatkin 1995) (Table 3.5). Although the "Tweed wolves" clustered with those from Algonquin Park, the R_{ST} value between the two groups was relatively high. In contrast, less structuring was apparent between those animals found to the west and northwest of the Park (Magnetawan region) and the Algonquin Park animals, which is consistent with the results from STRUCTURE. Surprisingly the animals from northeastern Ontario, in the area north of the French River and east of Georgian Bay and Lake Superior showed marked structuring with the gray wolves found in the adjacent Pukaskwa National Park, and much less with the more distant Algonquin Park *C. lycaon* population. Even more surprising was the lack of structuring of the northwestern Ontario animals and the animals in Algonquin Provincial Park. In both northwestern and northeastern Ontario there was little structuring between animals with Old World or New World mtDNA, suggesting a single population with both types of mtDNA segregating (Table 3.2). We interpret these data to suggest that although the wolves in Algonquin Provincial Park have been impacted by coyote genetic material in the past there is limited gene flow at present. This is in contrast to the connectivity between the northwestern, northeastern and Algonquin Park animals. The large genetic distance value between the Algonquin Park animals and those in northeastern and northwestern Ontario compared to the "Tweed wolves" and Algonquin Park wolves is probably due to the presence of gray wolf genetic material. Gray wolf genetic material is

Table 3.5. The estimate of R_{ST} for each geographic region. Values of less than 0.050 are indicative of higher levels of gene flow and are provided in bold.

	1	2	3	4	5	6
1. Algonquin	0.000					
2. Frontenac	0.113	0.000				
3. Magnetewan	**0.047**	**0.048**	0.000			
4. Northeastern Ontario	**0.018**	0.129	**0.029**	0.000		
5. Northwestern Ontario	**0.002**	0.130	**0.047**	**0.029**	0.000	
6. Pukaskwa National Park	0.254	0.365	0.278	0.171	0.198	0.000

far more divergent than the New World-evolved genetic material in the western coyotes and eastern timber wolves. This is supported by the presence of a high proportion of Old World mitochondria in these areas.

Taxonomic Relationships

In order to assess the taxonomic relationships of the Ontario groups and the homogeneity of the animals within the groups, we used an assignment procedure that was based on the likelihood of a genotype originating from one of three taxonomic groups (Wilson et al. 2000). We took control samples from three canis species, gray wolves from Northwest Territories, red wolves from the captive breeding program and western coyote from Texas and Ohio. Individual indices were developed between *C. lupus* and *C. lycaon* and *C. lycaon* and *C. latrans*. All three species formed individual ellipses (95% confidence) with no overlap (Figure 3.5A). Using these standards and 95% confidence intervals, we tested the distribution of the 6 geographic populations in Ontario. The "Tweed wolves" of the Frontenac Axis and the Magnetawan region showed considerable overlap with those of Algonquin Park animals and western coyotes (Figure 3.5B, 3.5C). This is consistent with the theory that the "Tweed wolf" is a hybrid between the western coyote and the eastern timber wolf. Algonquin wolves showed an overall different distribution of values from Northwest Territories wolves (Figure 3.5C), consistent with previous findings (Wilson et al. 2000). The majority of animals from Pukaskwa National Park did not overlap with Algonquin animals and were clustered most closely with the NWT gray wolf (Figure 3.5C). For animals in northeastern Ontario (Figure 3.5D) and northwestern Ontario (Figure 3.5D), although distributions were different, some animals

Figure 3.5. Two dimensional distributions of Individual Indicies based on *C. lupus /C. lycaon* and *C. latrans / C. lycaon* designations. **A.** Distribution of Northwest Territories gray wolves (open squares), red wolves (open circles) and western coyotes (open triangles from Texas and Ohio based on microsatellite allele frequencies from representative *C. lupus, C. lycaon* and eastern coyotes (*C. latrans*) samples. The log likelihood (Individual Index, Ii) of a genotype originating from three canis species was determined in these areas. 95% ellipses for each species is designated. **B.** Distribution of the log likelihood of a genotype originating from one of the three species determined for animals in the Frontenac Axis (closed circles) and Magnetewan regions (closed squares). **C.** Distribution of the log likelihood of a genotype originating from one of the three species determined for animals in Algonquin Provincial Park (closed triangles) and Pukaskwa National Park (closed diamonds). **D.** Distribution of the log likelihood of a genotype originating from one of the three species determined for Northeastern (black crosses) and northwestern Ontario (gray dashes).

A.

B.

C.
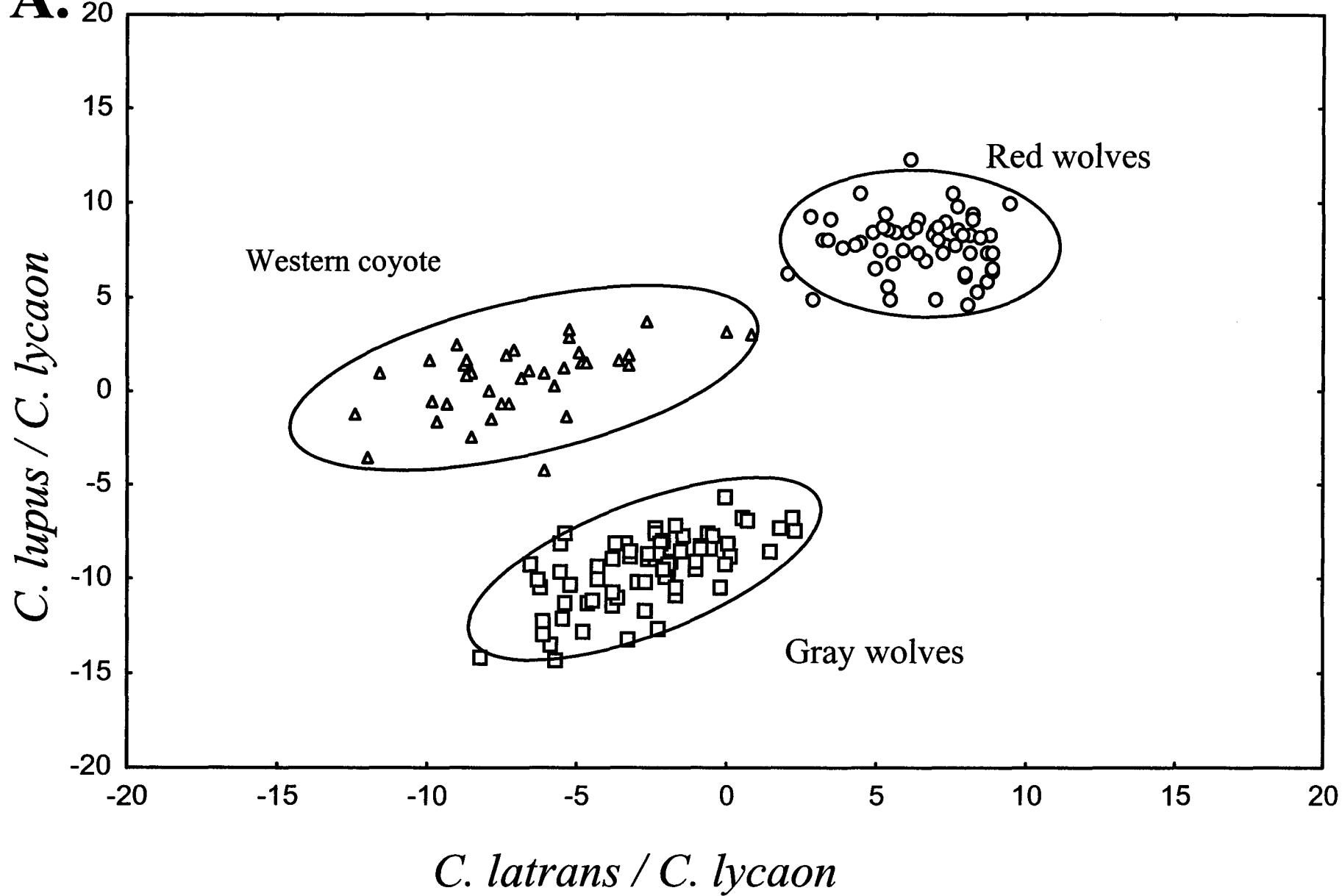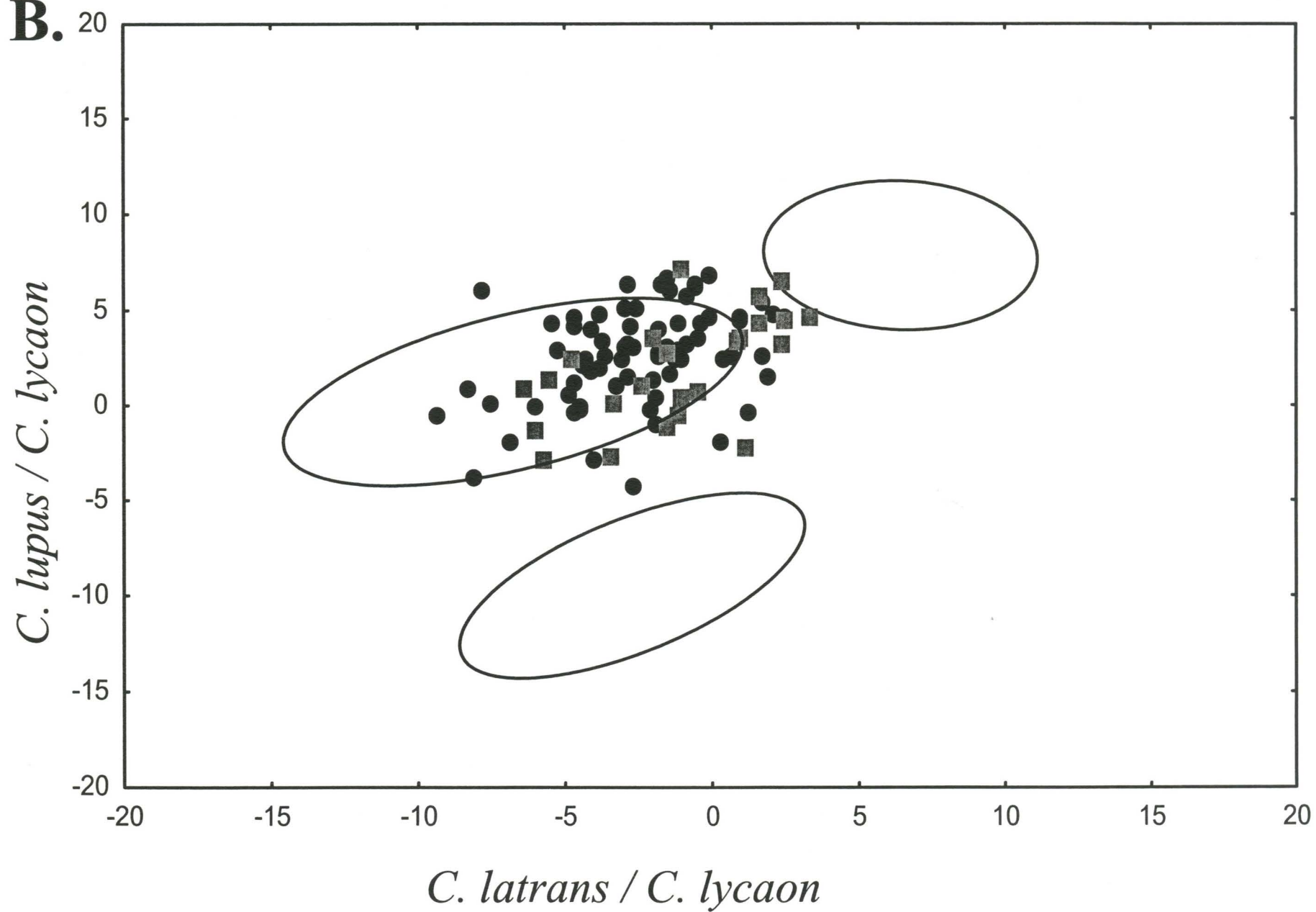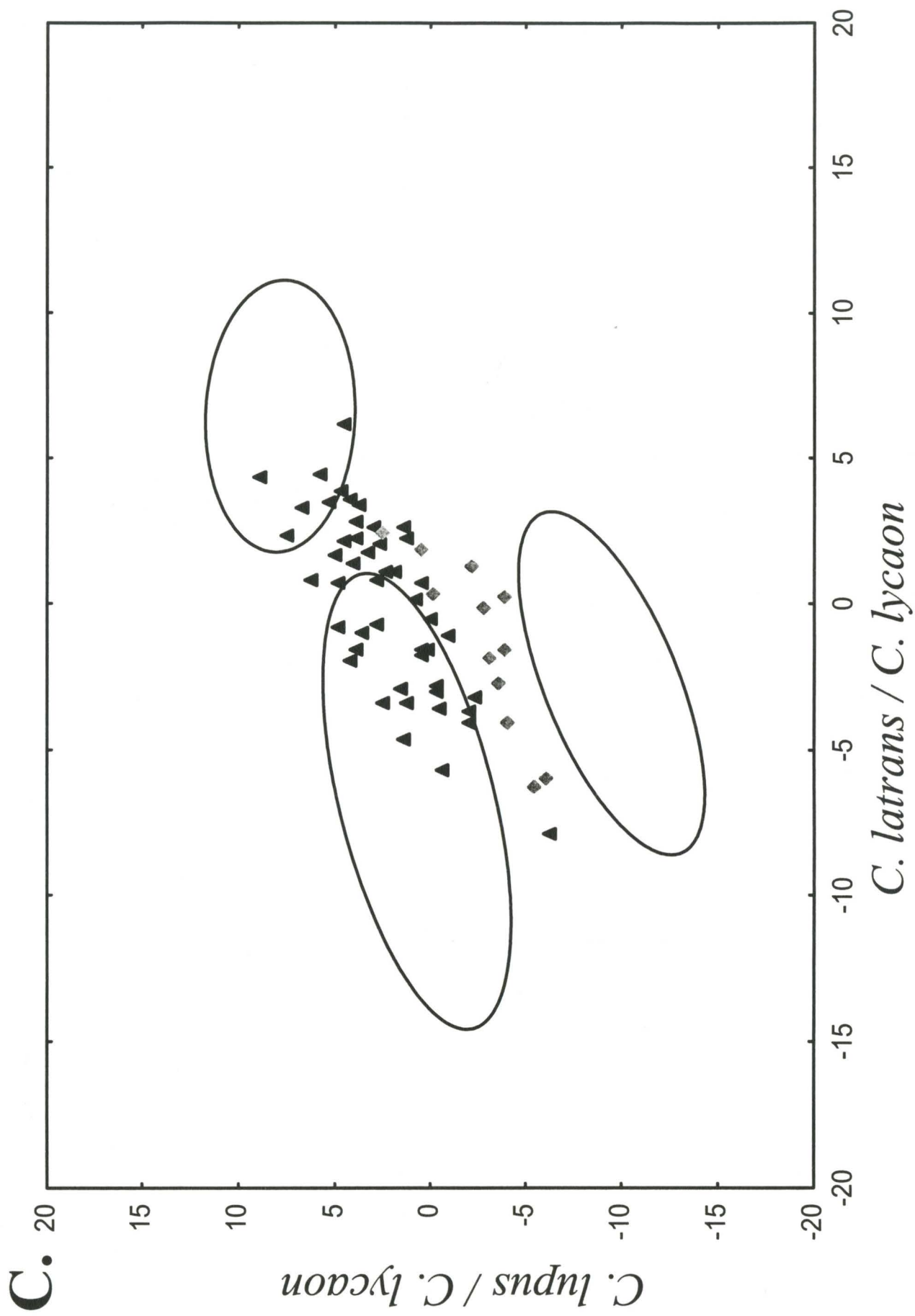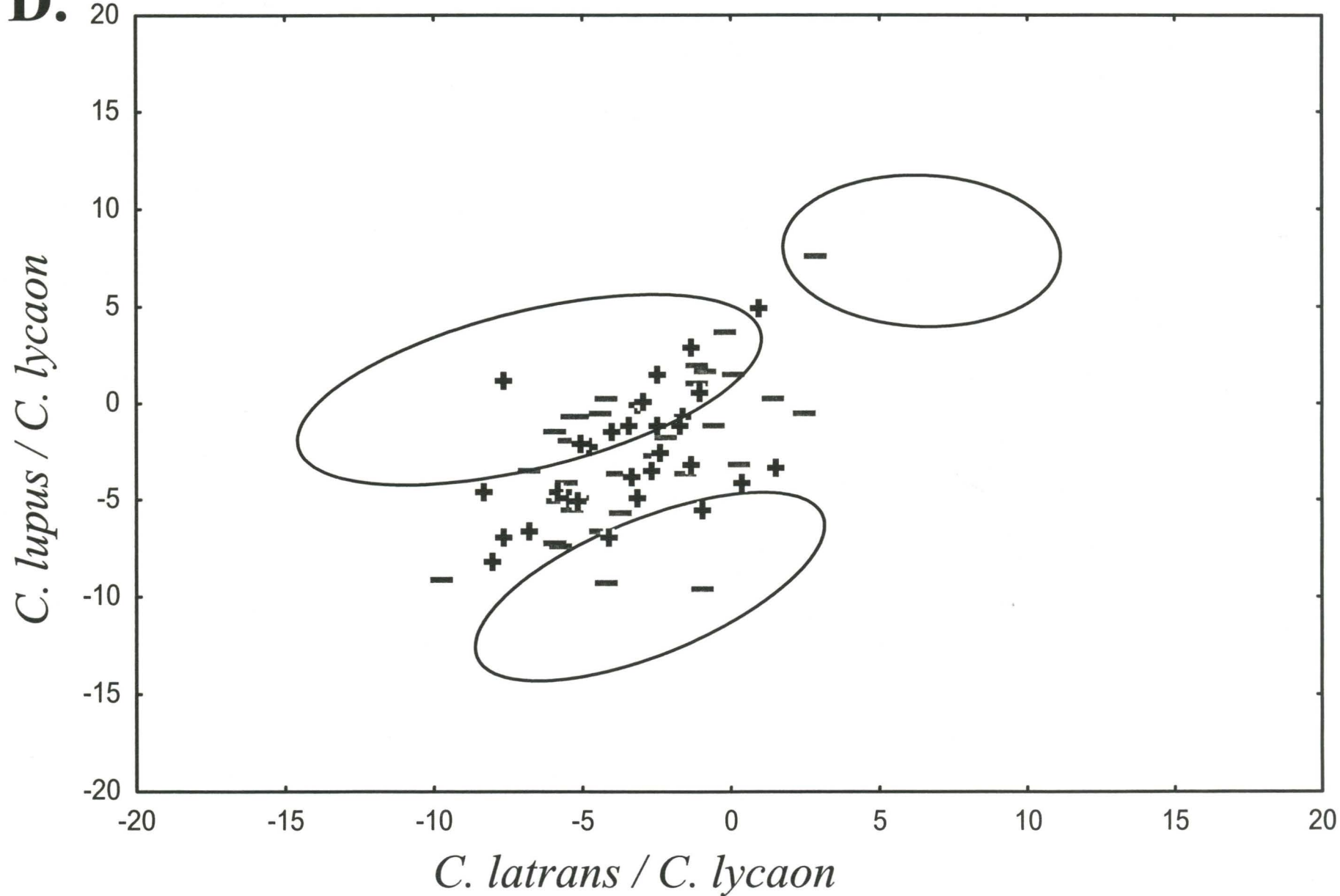
have genotypes resembling *C. lycaon* of Algonquin Provincial Park, although some appear to originate from *C. lupus* populations genetically similar to the Pukaskwa gray wolves. Low R_{ST} scores between, Algonquin Provincial Park and northeastern and northwestern Ontario provides evidence of gene flow (Table 3.5) consistent with the cluster analysis where animals from these regions appeared very similar. Unfortunately, we had few samples from the far north of Ontario to assess whether the Pukaskwa National Park animals were representative of gray wolves of the Hudson Bay Lowlands. One animal from the coastal region of Hudson Bay (Fort Severn) had a genetic profile similar to animals in Pukaskwa National Park.

Mitochondrial DNA in northern Ontario was a mixture of Old World (OW) and New World (NW). There was no correlation observed between OW mtDNA and index scores consistent with *Canis lupus* animals or NW mtDNA and index scores consistent with *C. lycaon* animals. All clustering and assignment tests suggest that *C. lycaon* animals are being impacted by *C. latrans* from the south and *C. lupus* from the north.

To assess the genome composition for the average animal in each of the geographic regions studied in Ontario, we calculated the average ancestry estimates from each of the four clusters (Table 3.6). In animals from the Frontenac Axis, 73% of the genome was characteristic of a "Tweed" type animal and only 19% was from an "Algonquin" or "*C. lycaon*" type genome. In contrast in Algonquin Park animals, 72% of the genome was of an "Algonquin" or "*C. lycaon*" type genome, whereas only 14% was of the "Tweed" wolf type animal. This is consistent with the lack of geneflow between

Table 3.6. Ancestry Estimates. The percent of genome composition for the average animal in each of the geographic regions studied in Ontario. This was calculated using ancestry estimates from each of the four clusters based on the program STRUCTURE. The numbers in bold represent the cluster type, which comprises the majority of the genome in that geographic region.

	Tweed type animal (Cluster 1)	Algoqnuin type animal (Cluster 2)	Northern Ontario type animal (Cluster 3)	Gray wolf type animal (cluster 4)
1. Frontenac Axis	**0.720 (0.323)**	0.190 (0.305)	0.041 (0.073)	0.035 (0.080)
2. Algonquin Park	0.137 (0.250)	**0.716 (0.338)**	0.093 (0.180)	0.052 (0.082)
3. Magnetewan Region	**0.401 (0.372)**	0.286 (0.323)	0.201 (0.275)	0.113 (0.215)
4. Northern Ontario	0.086 (0.227)	0.124 (0.247)	**0.478 (0.331)**	0.311 (0.288)
5. Pukaskwa National Park	0.010 (0.006)	0.091 (0.254)	0.104 (0.215)	**0.831 (0.314)**

these two regions. The Magnetawan and Northern Ontario animals had a mixture of ancestry, with 40% "Tweed" type in Magnetawan to only 9% in Northern Ontario. This is in contrast to the low "Ontario" type or "*C. lupus*" type genome found in the Magentawan region (11%) and the high amount in the northern Ontario samples (31%). The highest "Ontario" type or "*C. lupus*" type genome was present in the animals from Pukaskwa National Park (83%).

The genetic data show remarkable congruence with the "races" or "types" described by Kolenosky and Standfield (1975). The four clusters inferred by the non *a priori* assignment analysis are consistent with the four races or types described. In contrast to suggestion of Kolenosky and Standfield, there does appear to be a genetic cline between the Algonquin type animal and the Ontario type. However the absence of a pure *C. lycaon* in Ontario has made the cline between a *C. lycaon* impacted by coyotes (Algonquin type animal) and a *C. lycaon* impacted by *C. lupus* (North eastern and western Ontario animals) more difficult to assess. This is primarily due to the fact that the *C. lupus* material in *C. lycaon* animals is much more divergent from *C. lycaon* than the *C. latrans* material is from *C. lycaon*. Therefore genetic differences between a *C. lycaon* impacted by *C. lupus* and *C. lycaon* impacted by *C. latrans* appears greater than differences observed between *C. lycaon* and the Tweed type animal.

Discussion

Prior to European settlement, wolves occupied all of Ontario (Bates 1958) and primarily preyed on larger ungulates such as elk (*Cervus elaphus*), caribou (*Rangifer*

tarandus) and moose (*Alces alces*). Forested ecosystems were substantially altered as a result of logging and agriculture. These activities resulted in the decline of large ungulates such as elk and woodland caribou and also their gray wolf (*C. lupus*) predators and allowed the northern advancement of deer and eastern wolves (*C. lycaon*) and eventually coyotes (*C. latrans*). Changes in prey and habitat finally resulted in the elimination of wolves in southern Ontario (Standfield 1970). It is interesting to speculate that areas such as Algonquin Provincial Park were originally dominated by gray wolves preying on elk, caribou and moose and that the logging and associated human killing drove out the gray wolves and eliminated or reduced these large ungulates. As white-tailed deer moved into these areas they were followed by *C. lycaon* possibly originating from the Adirondacks and moving through the Frontenac Axis or from southern Ontario. The reduction of wolves from southern Ontario also allowed the spread of the coyote throughout the newly created farmland and the subsequent hybridization with *C. lycaon*.

The genetic data presented supports the hypothesis that the "Tweed wolf" as described by Kolenosky and Standfield (1975) and Kolenosky and Schmitz (1985) is a hybrid between the "Algonquin" type wolf and the coyote (*C. lycaon* x *C. latrans*). However, in contrast to Kolenosky and Standfield, recent genetic evidence (Wilson et al. 2000) suggests that these hybrids originated from inter-breeding between two North American evolved Canis species, *C. lycaon*, representing the eastern timber wolf and red wolf, and the coyote, *C. latrans*. The absence of the gray wolf in this hybridization explains the anomaly of the lack of inter-breeding between western coyotes and gray wolves (Roy et al. 1994, Wilson et al. 2000). As Kolenosky and Schmitz (1985) alluded

to, the absence of a "pure" coyote in southern Ontario is apparent immediately south of Algonquin Park and into the Frontenac Axis. This hybrid eastern wolf/coyote is extremely adaptable to both agricultural and low-density forested habitats. Despite the high numbers of the "Tweed" animals, southeast of Algonquin Park, the data suggests barriers to gene flow exist by maintaining larger wolf-like animals within the Park. Despite limited gene flow from the Frontenac Axis, the high level of genetic variation in Algonquin Park (Table 3.2) is supported by the gene flow from the Magnetawan region, northeastern Ontario and potentially from Quebec. Although the Algonquin Park population numbers less than 200, evidence suggests it is part of a larger metapopulation that includes animals from northeastern and northwestern Ontario and Quebec (Grewal et al. *in Prep*). The low R_{ST} values between the northeast and northwest animals suggest there is substantial gene flow between both regions of the province. This supports the conclusion the population of *C. lycaon* in Ontario is large, numbering in the thousands rather than the hundreds. The eastern wolf ranges in size from smaller animals in Algonquin Provincial Park to larger animals in northeastern and northwestern Ontario (Kolenosky and Standfield 1975). This cline is likely related to the introgression of more coyote genetic material in the south and more gray wolf genetic material in northern Ontario. Introgression of genes into *C. lycaon* animals may further be influenced by selection based on factors such as prey size (Hillis 1990, Mulders 1997). *C. lycaon* within Algonquin Park prey predominantly on white-tailed deer and beaver (Forbes & Theberge 1996). With the ecological changes in Algonquin Park from a high density of deer in the 1960s to the present lower densities and the highest moose densities in the

province (Whitlaw and Lankester 1994), a selection for larger animals that can utilize moose more effectively might occur in the future. The connectivity of the Algonquin Park population to the northern animals may facilitate this natural evolution.

Pukaskwa National Park maintains a small population of gray wolves (*C. lupus*) that prey on moose, and appear to be surrounded by the larger "Algonquin type" animals in patchy habitat that contains moose and white-tailed deer. The high structuring value between the gray wolves in Pukaskwa and the eastern timber wolves suggests a predominantly *C. lupus* population with limited gene flow to *surrounding C. lycaon*.

The broad band across northeastern and central Ontario, which Kolenosky and Standfield (1975) described as the area where the "Ontario" and "Algonquin" types meet, but in which interbreeding was apparently absent, now appears to contain hybrid wolves (Table 3.6). However, the hybrids still appear to be primarily "Algonquin" genotypes suggesting the hybridization between *C. lupus* and *C. lycaon* is more restricted than that between *C. lycaon* and *C. latrans* to the south of Algonquin Park.

Due to our reliance on fur samples from commercial fur houses for many of our more northern samples we have few samples to allow us to assess animals in the Hudson Bay lowlands and the coastal regions of Hudson and James Bay. Of interest is the single animal from Fort Severn on the Hudson Bay coast that was assigned as originating from Pukaskwa National Park. This suggests there may not be a separate gray wolf subspecies, *C. l. hudsonicus* in the Hudson and James Bay coastal areas. This is consistent with the Pukaskwa population representing a remnant population of gray wolves, while most of the population moved further north or was extirpated.

In northwestern Ontario, populations appear to be genetically related to animals in Algonquin Provincial Park. There appears to have been less hybridization with coyotes and an absence of Tweed wolves in this area. Animals have been classified genetically as *C. lycaon* based on the structure analysis using the microsatellite DNA profiles but the presence of a number of animals with gray wolf mtDNA haplotypes is evidence of past and perhaps present hybridization with *C. lupus*. The Minnesota and Wisconsin wolves are most likely the same as the animals in northwestern Ontario. Increases in wolf numbers in Minnesota have led to moves to de-list the gray wolf as endangered in the U.S. We would urge caution until classification of these wolves is clarified.

In summary, the genetic data support the hypothesis that the "Tweed wolf" is a hybrid between the coyote and eastern timber wolf (*C. latrans* and *C. lycaon*). The eastern timber wolf appears to be a North American-evolved species closely related to the red wolf and represents the "Algonquin" type described by Kolenosky and Standfield (1975). We suggest it retain its original taxonomic designation of *C. lycaon*. In northeastern and northwestern Ontario, *C. lycaon* has hybridized with the gray wolf (*C. lupus*) and is larger than the animals found in Algonquin Provincial Park. The populations in northeastern and northwestern Ontario appear to be genetically connected with the Algonquin Provincial Park population and Quebec populations (Grewal et al. *in Prep.*) and the total number of animals may be in the thousands. Pukaskwa National Park contains a small isolated population of *C. lupus* that might represent the original "Ontario type" described by Kolenosky and Standfield (1975). As a result of poor sampling in northern Ontario we have not resolved the genetics of the wolves in Hudson Bay

lowlands or the coastal regions of Hudson and James Bay. The single animal from the Hudson Bay coast resembled animals from Pukaskwa suggesting that there may be only one gray wolf, *C. lupus*, subspecies in Ontario. The higher number of *C. lupus* animals in northeastern Ontario suggests the beginning of the present-day boundary between *C. lycaon* and *C. lupus* in this region. The equivalent boundary in northwestern Ontario may lie farther north as fewer *C. lupus* animals were detected in this region. The absence of a "Canis soup" in western North America appears to be attributed to the absence of *C. lycaon,* which readily hybridizes with coyotes and can hybridize with gray wolves, thus mediating gene flow among the three species.

Acknowledgements

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Preface

This chapter was written with co-authors and formatted for submission to a peer-reviewed journal and is therefore a self contained manuscript with an individual reference section.

Chapter 4: A genetic assessment of the packs of the Eastern Timber Wolf, *Canis lycaon* in Algonquin Provincial Park and their relationship to surrounding animals.

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Status: Submitted to the Journal of Mammology for review.

Contribution: Autosomal microsatellite and mitochondrial DNA data collection done by S.K.G. Data analysis and writing of this paper was done by S.K.G. Research was conducted under the supervision and guidance of B.N.W.

Chapter 4

A genetic assessment of the packs of the Eastern Timber Wolf, *Canis lycaon* in Algonquin Provincial Park and their relationship to surrounding animals

ABSTRACT

Recent genetic data indicate the eastern timber wolf is not a subspecies of the gray wolf but a North American-evolved wolf similar to the red wolf, *Canis rufus*, and closely related to the coyote, *Canis latrans* and it has been proposed it be designated *Canis lycaon*. The largest protected area containing this wolf is Algonquin Provincial Park in Ontario, which is bounded to the south by areas containing the "Tweed wolf" or eastern coyote, a western coyote / eastern wolf hybrid. We assessed the relationships of the animals in the Park using DNA profiles that comprised the genotype at 17 autosomal and 4 Y-linked microsatellite loci and the mitochondrial DNA control region. These profiles were used to establish maternity, paternity and kin relationships for 102 animals that were studied from 24 packs over a 12-year period. The data do not support the hypothesis that a pack comprises an unrelated breeding pair and their offspring. Some of the unrelated individuals in the packs were identified as immigrants to the Park and showed no evidence of breeding. Using genotypes at 8 microsatellite loci, the relationship of the Park wolves to the surrounding animals was assessed. Relatively high genetic structuring between the Park animals and the "Tweed" wolves to the southeast ($R_{ST} = 0.114$) was identified suggesting introgression of coyote genetic material is not a present concern. There is evidence of gene flow with animals to the west ($R_{ST} = 0.057$), northeast ($R_{ST} = 0.036$) and

northwest ($R_{ST} = 0.069$) and this coupled with the high genetic diversity, suggests that the Park animals are not an island population, but the southern part of a larger metapopulation of *C. lycaon*, that stretches from Quebec to Manitoba and which includes Minnesota, Wyoming and Michigan.

INTRODUCTION

Recent genetic data (Wilson et al. 2000) have been used to suggest that the eastern (Canadian) timber wolf, is not a subspecies of the Eurasian evolved gray wolf, but is the same species or a close relative of the red wolf, *Canis rufus* found in the southern United States. It has been proposed by Wilson et al. (2000) that the eastern timber wolf be given the original taxonomic designation of *Canis lycaon* (Miller 1912). Both taxa are thought to have a common origin evolving in North America, with the western coyote (*Canis latrans*) diverging from the lineage 150,000 – 300,000 years ago. This close evolutionary relationship is consistent with their ability to readily hybridize with western coyotes and the absence of hybridization between western and northern gray wolves and western coyotes. A significant number of protected packs of the eastern timber wolf are found in Algonquin Provincial Park. However a number of threats to the persistence of these packs have been identified including human caused mortalities when wolves leave the park and potential gene swamping from coyotes.

Primarily a heavily forested area in central Ontario, Algonquin Provincial Park borders the Frontenac Axis to the southeast and the Magnetawan region to the west. The Magnetawan region contains forested areas similar to Algonquin Park together with

developed farmland. The Frontenac Axis, which lies within the Canadian Shield comprises patchy areas of mixed forest (Sears 1999) and is considered a potential corridor to the Adirondacks in New York. To the north of Algonquin Park, in northeastern Ontario and Quebec a transition from mixed to boreal forest occurs.

Prior to European settlement in the 17th century, gray wolves were thought to have occupied all of Ontario (Bates 1958) preying primarily on larger ungulates such as elk (*Cervus elaphus*), moose (*Alces alces*) and caribou (*Rangifer tarandus*). Following the establishment of the Hudson Bay Company in 1670 and intense logging in the late 1800s, significant declines in ungulates and wolves occurred in south and central Ontario (Peterson 1955; Franzmann and Schwartz 1998). One region significantly affected after 1840 was Algonquin Provincial Park, which underwent intense logging and wolf control programs. The reduction in gray wolves and deforestation probably facilitated the movement of white-tailed deer and the smaller eastern timber wolves from the south at the end of the 19th century.

At the beginning of the 20th century western coyotes entered southern Ontario and probably hybridized with residual pockets of eastern wolves to form the eastern coyote which rapidly spread further eastward (Moore and Parker 1992) to occupy the Frontenac Axis and Magnetewan regions. As a result, these areas contain a diverse range of sizes of wolves and coyotes for which the term "canid soup" was coined. Kolenosky and Standfield (1975) recognized four "types" or "races" of wolves in Ontario. Along the coasts of the James and Hudson Bay the gray wolf subspecies *Canis lupus hudconicus* was identified. In the boreal forests of the Hudson Bay Lowlands and the deciduous

forests of the upper Great Lakes, a second gray wolf subspecies *C. l. lycaon* was identified. This subspecies was further separated into two types of wolves, the "Ontario type" and the Algonquin type" based on the described habitat and prey base of moose and deer respectively. The final "type" or "race" of wolf identified was the "Tweed wolf" which appears to have resulted from the hybridization of the "Algonquin type" with eastern coyotes. This mating may have been promoted by the implementation of wolf control programs by Algonquin Provincial Park rangers up until the 1960s.

Harsh winters in the 1970s substantially reduced the deer population in and around Algonquin Park. At the same time the Park was reverting to an ecosystem, closer to that found in the early 19th century, making it more suitable for moose than deer (Peterson 1955; Franzmann and Schwartz 1998). In the early 1980s, to compensate for the decline of deer, the Ontario Ministry of Natural Resources fed deer in wintering yards to the southeast of the park. This may have promoted deer migration from the Park (Forbes and Theberge 1995; Forbes and Theberge 1996).

The packs on the eastside of Algonquin Provincial Park have been the subject of an intense study involving the radio collaring of 150 wolves over 12 years. It has been estimated that in winter there are 170-200 animals in 30-35 packs (Forbes and Theberge 1996). This estimate is lower than that for the 1960s of approximately 300 animals (Pimlott et al. 1969), which was at that time suggested to be the carrying capacity of the Park. Migration of Algonquin wolves, 15-70 km (Forbes and Theberge 1995, 1996) each winter to the deeryard located 13 km southeast outside the Park (Forbes and Theberge 1995) has been documented. This migration appears to result in the high mortality

observed (50 animals per year), of which 60% is a result of human activity outside the park boundary (Theberge and Theberge 1997). A number of townships around the Park recently imposed limitations on hunting and trapping and the Algonquin Wolf Advisory Group (AWAG) has recommended further restrictions (AWAG 2001).

Early studies on the dispersal and social structure of wolves were primarily based on limited observations on gray wolf packs in Alaska (Murie 1944; Rausch 1967), and the Northwest Territories (Fuller and Novakowski, 1955), and eastern timber wolves from Isle Royale (Jordon et al. 1967), Minnesota (Olson 1938; Mech 1970) and Ontario (Kolenosky and Johnston 1967; Pimlott et al. 1969). These data were used to formulate a generally accepted model of a wolf pack as a breeding pair (alpha male and alpha female) and their offspring. The subordinate animals were thought to be offspring from the previous year. Little consideration was given to possible significant differences in pack structure between northern gray wolves and eastern timber wolves and deviations from the model were usually considered to result from odd and unusual events. Recent studies have used more intense and long term radio-collaring and tracking techniques (Van Ballenberghe et al. 1975; Fritts and Mech 1981; Forbes and Theberge 1996) and genetic profiles (Wayne et al. 1991; Laikre and Ryman 1991; Lehman et al 1992; Meier et al. 1995; Wayne et al. 1995; Forbes and Boyd 1996; Smith et al. 1997). These studies suggest a more complex pack structure resulting from pack formation by wolves dispersing from nearby territories, the splitting of existing packs and frequent pack mergers or adoptions. Pack fusion or mergers are similar to adoption except that more than one animal from a pack (which may have undergone partial decimation) merges with

a smaller pack to form a new family unit. This unit may use both territories temporarily until settling into one area. In terms of genetics, a higher level of similarity has been observed among adjacent eastern wolf packs in Minnesota than gray wolf packs in Alaska or the Northwest Territories (Lehman et al. 1992; Meier et al. 1995; Wayne et al. 1995). Although habitat fragmentation was considered to be responsible for this, species differences between the gray wolf and eastern timber wolf need to be considered (Wilson et al. 2000).

As Algonquin Provincial Park contains the most intensely studied group of packs of the eastern wolf, one of the objectives of this genetic study was to test previous hypotheses of pack composition. We attempted to answer the following questions: Is a pack composed of an unrelated breeding pair and their offspring? If not, what is the origin and nature of the unrelated subordinate animals? Is a pack and its territory more often passed from father to son or mother to daughter? To address these questions we used three different types of genetic markers; 1) seventeen autosomal microsatellite loci; 2) the mitochondrial DNA control region; and 3) four Y- chromosome microsatellite loci located below the pseudoautosomal region. We also looked at the relationship of the park animals to those outside with particular reference to gene swamping from coyotes and genetic structuring and gene flow with other groups of eastern wolves to the north.

MATERIALS AND METHODS

Samples and DNA extraction

Over the 12-year field study (Forbes and Theberge 1995, 1996; Theberge and

Theberge 2000) 150 animals from 35 packs in Algonquin Provincial Park were live

trapped and radio-collared between 1987-1999 (Fig. 4.1a). Ninety-seven, blood and

tissue samples from radio-collared animals plus an additional 5 non radio-collared

animals from 24 packs were extracted (Grewal et al. in prep.). To estimate the proportion

of individuals sampled from each pack (P) over the twelve years of study, we multiplied

the number of packs (Np) sampled by the average number of animals (N/12) per pack and

added that to the number of animals recruited (births and migration) which has been

estimated at 30% per year (Theberge and Theberge 2000).

P (individuals sampled/pack) = (Np * N/12) + (0.30 * 12)*100

The animals in regions of Ontario surrounding Algonquin Provincial Park (Fig.

4.1b) are separated into; the Frontenac Axis (n = 74), the Magnetawan Region (n = 26)

and northeastern Ontario (n = 33) (Grewal et al. pers. comm.). There were also samples

from two groups from Quebec; one south of the Temiscamingue-Abitibi region (n = 13)

and the other, La Verendrye Reserve (n = 13) (Fig. 4.1b).

Genetic Markers

Mitochondrial DNA Control region Sequence Analysis.- Primers described in

Wilson et al. (2000) were used to amplify the control region of the mitochondrial DNA

for specific haplotype identification. The control region was amplified in a total reaction

volume of 25µl per tube using 25ng of genomic DNA, 200 µM dNTPs, 1x amplification

buffer, 2 mM MgCl₂, primers 1 and 2 (0.2 µM) and 0.5 units of Taq polymerase (Gibco

BRL, Burlington, Ontario, Canada). Products were amplified under conditions described

Fig. 1.- Map of sample locations, a) Distribution of the 24 packs studied on the east side of Algonquin Provincial Park between 1987-1999. Pack boundaries are indicated by circles and were established from the radio-collaring data (Forbes and Theberge 1995). The star represents the Round Lake deer-yard, b) Distribution of the 6 groups of animals studied in the provinces of Ontario and Quebec. Frontenac Axis ($N = 74$), Magnetawan Region ($N = 26$), Algonquin Provincial Park ($N = 102$), Northeastern Ontario ($N = 33$), Abitibi-Temiscamingue region ($N = 13$), La Verendrye Reserve ($N = 13$). The dotted lines represent boundaries for the respective geographic sampling locations.

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Traverse

Pretty Mathews Hardwood

Grand

Military

Jackpine Grand

Lavielle Jocko MacDonald

Basin

Redpole Foys Acorn

Annie Killaloe
Bay Cybulski Limestone

Byers

Eastgate Poplar
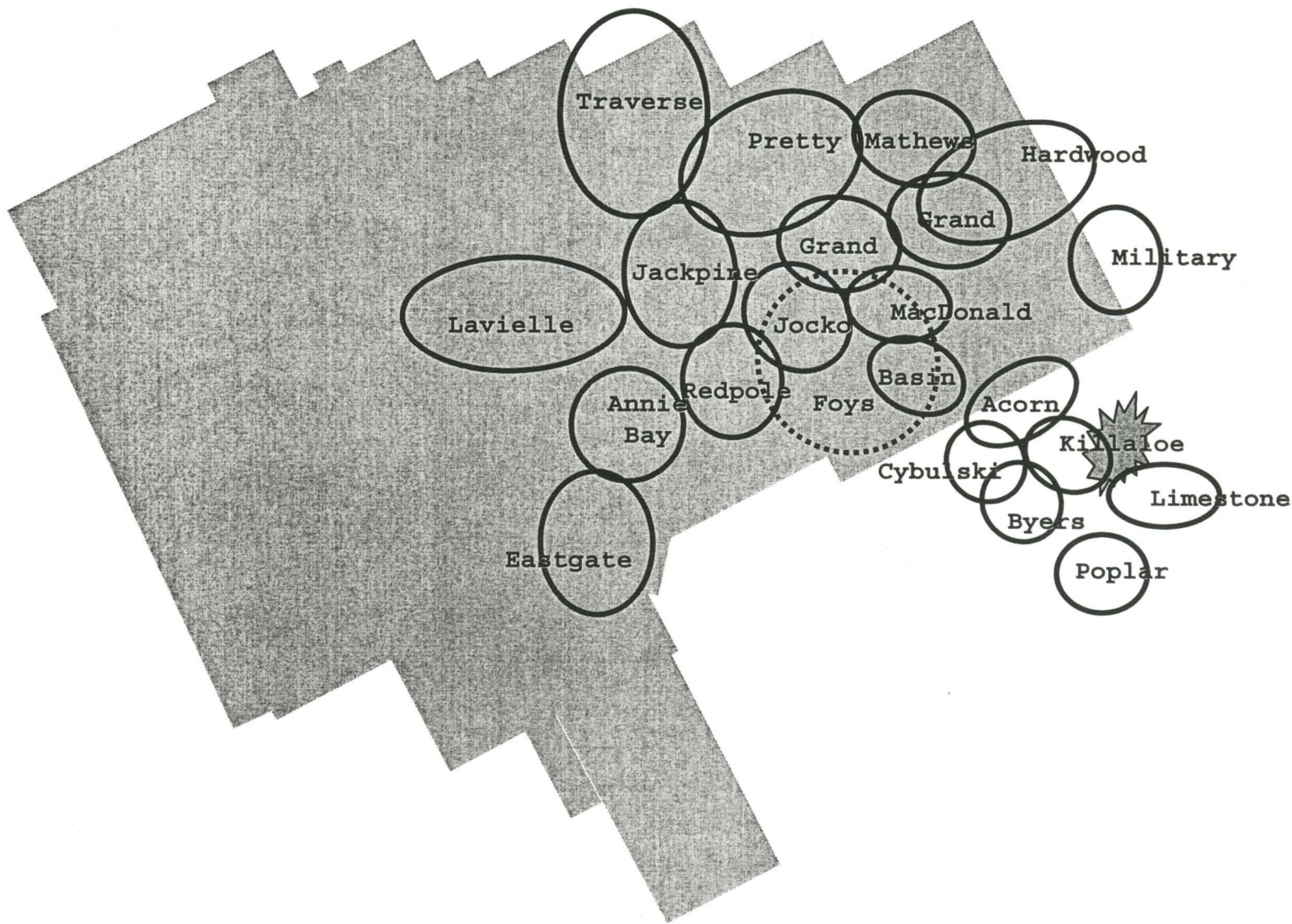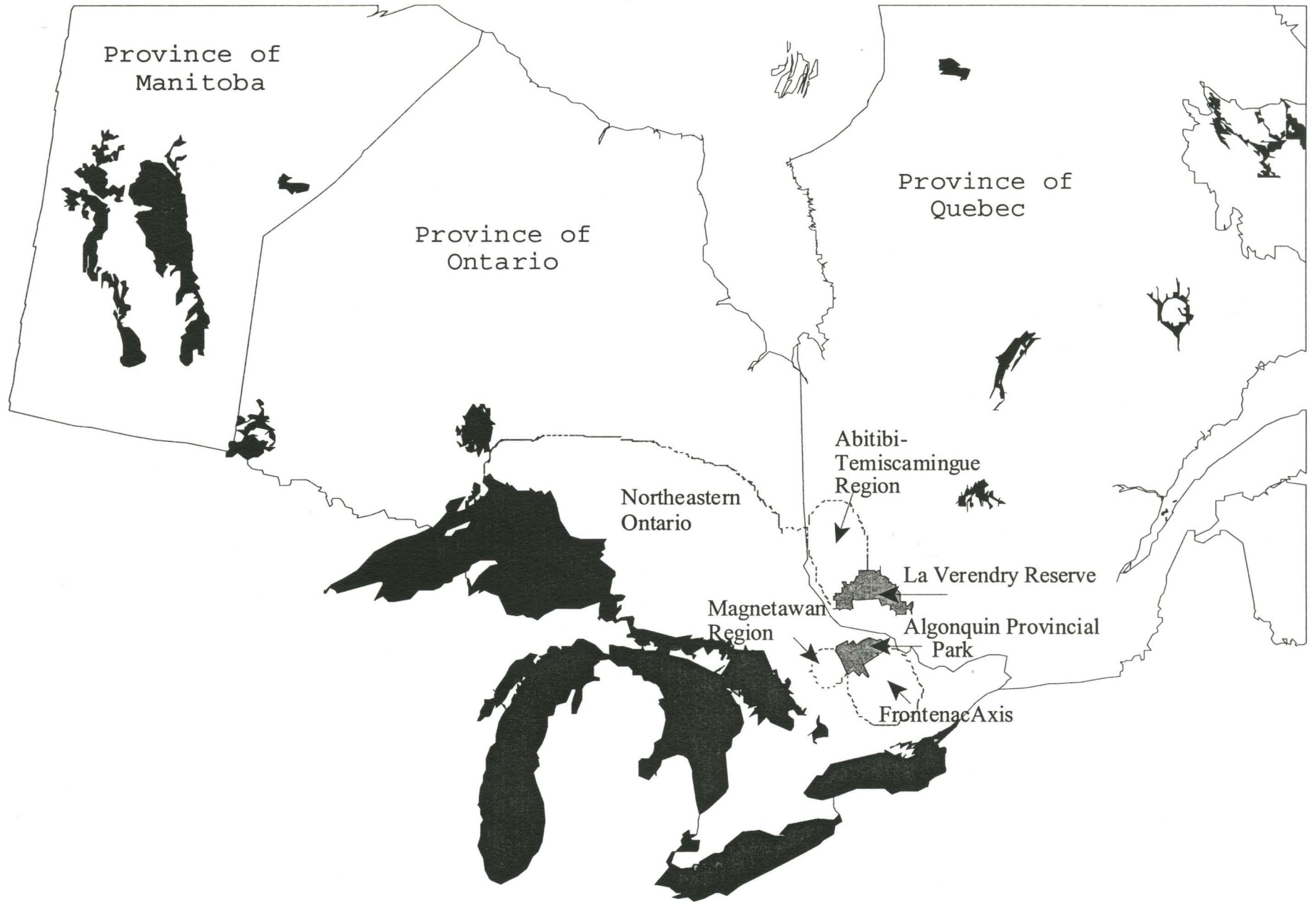
Province of
Manitoba

Province of
Ontario

Province of
Quebec

Abitibi-
Temiscamingue
Region

Northeastern
Ontario

La Verendry Reserve

Magnetawan
Region

Algonquin Provincial
Park

Frontenac Axis

in Wilson et al. (2000). Products were sequenced using the ABI 377 Sequencer (Applied

Biosystems, Foster City, California). A set of samples, which included a representative

set of all the haplotypes were used as controls to further screen individuals using single

stranded conformation polymorphism (SSCP) analysis. Amplified products were

electrophoresed through a non-denaturing acrylamide gel (5% acrylamide [59 acrylamide:

1 bisacrylamide], 10% glycerol and 0.5X TBE) for 16 hours at 4^0C. Prior to loading, PCR

products were mixed with 20% formamide, then denatured for 10 minutes (95^0C) and

placed on ice for 5 minutes. New haplotypes identified by SSCP were sequenced using

the ABI 377 Sequencer (Applied Biosystems, Foster City, California).

Autosomal Microsatellite Analysis.-The 17 microsatellite loci were amplified in a

total reaction volume of $10\mu l$ using 25ng of genomic DNA, 200 μM dNTPs, 1x

amplification buffer, 2 mM $MgCl_2$, unlabeled primers R and F (0.2 μM and 0.18 μM),

radioactively labeled (γ^{33}P-dATP (ICN) F primer (0.02 μM), 1 μg of Bovine Serum

Albumin (BSA) (Amersham Pharmacia, Baie d,Urfe, Quebec, Canada) and 0.5 units of

Taq polymerase (Gibco BRL, Burlington, Ontario, Canada). Products were amplified

under conditions described by Wilson et al. (2000) and loading onto a 6% sequencing gel

containing 50% (w/v) urea. A control sequencing reaction of phage M13 DNA was run

adjacent to the samples to produce size markers for the microsatellite alleles. The bands

were visualized by autoradiography.

Y-Chromosome Microsatellite Analysis.- Using the above methods Y-chromosome

microsatellite primer sets (MS34 and MS41) characterized by Olivier et al. (1999),

amplified 4 loci (Karmi et al., in prep.). Haplotypes were established using the combination of alleles at the four loci amplified.

Statistical Analysis

Genetic Variation.-Allele frequencies, expected heterozygosity (H_E) and allelic diversity (A) were calculated at 8 loci using the program Cervus (Marshall 1998). The inbreeding coefficient (F_{IS}) (Weir and Cockerham 1984) was calculated using the program Genetix 4.02.

Parentage.- Three data sets were employed to assess parent-offspring relationships. First, genetic exclusion of a putative parent was determined if it did not share at least on allele at each of the 17 loci with the offspring (Fig. 4.2). The second level involved the mtDNA and Y-chromosome markers. A mother was excluded if her mtDNA haplotype was not identical to her putative offspring and a father was excluded if his Y-chromosome haplotype was not identical to that of his putative son (Fig. 4.2). The third approach was based on the field data so that the putative parent had to be alive the same year as the birth of the offspring and not exceed 10 years of age. This allowed for field age identification errors and is based on the assumption that eastern wolves may breed after one year of age but not after 10.

Kin Relatedness.- Allele frequencies were calculated for 17 microsatellite loci from the genotypes of 73 Algonquin Provincial Park wolves. In order to avoid bias in

Fig. 4.2.- An example of pack relatedness using genetic criteria. A putative father and his four offspring were identified because of one allele at each locus in the father was passed down to each offspring (bold). Uninformative loci in each offspring are identified when both alleles in the offspring are italicized. Therefore it is uncertain which allele in the offspring came from the mother and which allele came from the father. The Y chromosome haplotype AA is also passed on to the male progeny. Based on the alleles not contributed by the father and the maternally inherited mitochondrial haplotype, C22, one putative mother is possible and although the female, which mated with the putative father, is unknown, the genotype of the mother can be predicted. A slash (/) indicates that the putative mother may have either allele. The mother's genotype suggests that all four offspring are full sibs.

? — Jocko 10

Locus	Allele 1	Allele 2
377	162	164
225	163	167
109	143	149
172	144	144
250	135	137/141
123	143	153
200	215	217
204	200	200
147	172	178
606	170	172
253	109	117
442	164	166
410	98	114/118
383	167	167
2010	230	234
Cph11	159	173/177

Mt haplotype = C22
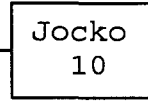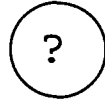
Locus	Allele 1	Allele 2
377	156	164
225	167	167
109	143	143
172	144	144
250	137	141
123	147	153
200	215	215
204	200	200
147	168	172
606	170	170
253	109	117
442	164	166
410	114	118
383	167	167
2010	226	230
Cph11	173	177

Y haplotype = AA
Mt haplotype = C22

Jocko 11
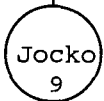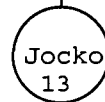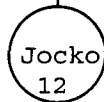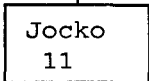
Allele 1	Allele 2
156	164
167	167
143	143
144	144
137	141
147	153
215	215
200	200
168	172
170	170
109	117
164	166
114	118
167	167
226	230
173	177

Y haplotype =AA
Mt haplotype =C22

Jocko 12

Allele 1	Allele 2
156	162
163	167
143	149
144	144
135	141
143	147
215	215
200	200
168	178
170	170
109	109
164	164
98	118
167	167
230	230
159	173

Mt haplotype =C22

Jocko 13

Allele 1	Allele 2
162	164
163	167
143	149
144	144
135	141
153	153
215	217
200	200
172	178
170	170
109	117
164	166
98	118
167	167
226	234
159	177

Mt haplotype =C22

Jocko 9

Allele 1	Allele 2
156	162
163	167
143	149
144	144
135	137
153	153
215	215
200	200
172	172
170	172
109	109
166	166
98	118
167	167
226	230
159	177

Mt haplotype =C22

calculating allele frequencies, the genotypes of all known offspring were omitted from the allele frequency calculation. Using KINSHIP 1.2 (Queller and Goodnight 1989) three simulations were performed: 1000 randomly generated pairs of unrelated individuals ($r = 0$); 1000 pairs of half sibs ($r = 0.25$) and 1000 pairs of full sibs ($r = 0.5$). From the simulation data, the mean and standard deviation of unrelated, half sib and full sib distributions were calculated. Using the program STATISTICA, confidence levels of 95% were calculated for each distribution in order to classify dyads (Fig. 4.3). The upper 95% confidence level for unrelateds was 0.238, for half sibs 95% of observations were found between –0.220 and 0.518 respectively and the lower 95% confidence level for full sibs was 0.269. Individuals with R-values greater than 0.518 were identified as full sibs (Fig. 4. 3). Unless specific relationships were being tested, individuals with R-values > 0.238 were identified as being related. Therefore any dyads with R-values falling within the overlap of unrelated animals and half sibs were identified as unrelated. The index weights each allele inversely by its frequency in the population, so that rare alleles are given a relatively higher weighting. Relatedness values can range from -1.0 to 1.0, in which a negative value means that the dyads share fewer alleles than the average population.

Genetic structuring and immigration.- Genetic structuring (ϕ) between populations based on mitochondrial DNA and Y-chromosome haplotypes was estimated using the program AMOVA (Analysis of Molecular Variance, version 1.55; Excoffier et al. 1992). Genetic structuring between populations based on microsatellite data was estimated using R_{ST} (Slatkin 1995) and F_{ST} (Weir and Cockerham 1984). Levels of significance for the pairwise R_{ST} values were calculated following 1000 bootstraps and permutations of the

Fig. 4.3.- Histograms of simulated relationships as generated using KINSHIP (Queller and Goodnight 1989). Shown are simulated dyads based on 1000 randomly generated pairs of unrelated individuals (black bars); 1000 pairs of half-sibs (horizontal bars) and 1000 pairs of full-sibs (clear bars). Curves are normal distributions for each simulated relationship created by STATISTICA. Arrows delineate the range under which 95% of all observations fall for unrelateds (diamond headed arrow), half-sibs (triangle headed arrows) and full-sibs (oval headed arrows).
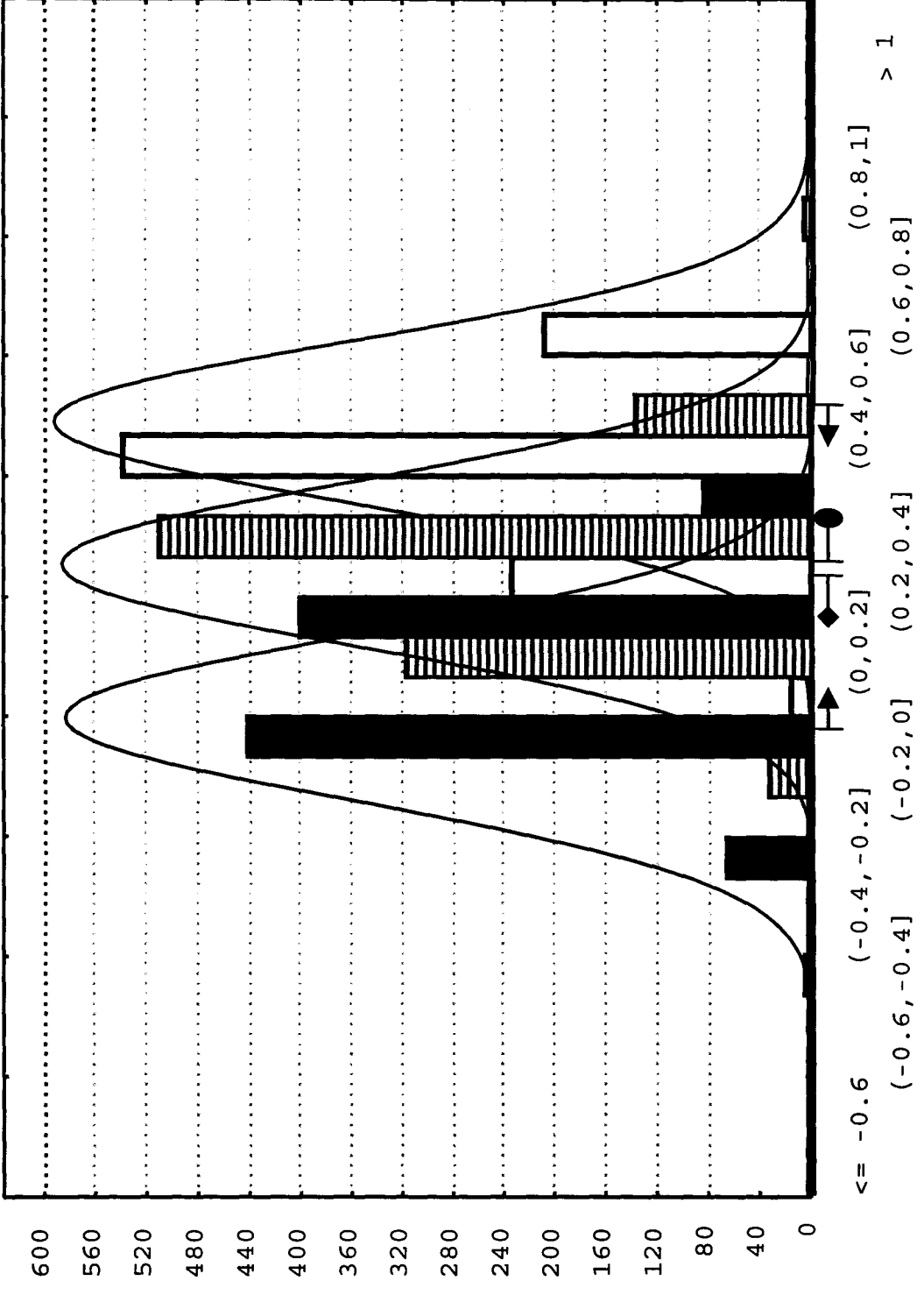
data using the computer program R_{ST}CALC (Goodmann 1997). Theta was calculated for each population pair and its significance from zero was assessed with 1000 permutations of the data using the program Genetix 4.02. Immigrants into Algonquin Park were identified using the program STRUCTURE (Pritchard et al. 2000). This provides a conservative minimum estimate as it does not identify immigrants from areas with similar genotypes. Confidence levels of 95% were used to determine if an animal was an immigrant.

RESULTS

Algonquin Park Samples

The 102 samples from Algonquin Provincial Park animals (Forbes and Theberge 1995, 1996; Theberge and Theberge 2000) used for genetic analysis represented 24 of the 35 packs studied over a 12 year period (Tables 4.1 and 4.2) (Fig. 4.1a). The study focused primarily on the eastside of Algonquin Provincial Park, with pack boundaries established from the radio-collaring data (Forbes and Theberge 1995). Males and females were equally sampled (Table 4.1). The presence or activity of a pack in a given year was defined by the presence of at least one collared and sampled individual from that pack. Nine of the 24 packs from which radio-collared animals were sampled had only one member sampled for genetic analysis. The proportion of individuals sampled over the 12 years was estimated from the average number of animals per pack and a recruitment of 30% per year (Theberge and Theberge 2000). We estimated a total of 462 different animals would have been present in the 24 packs over the span of 12 years. Therefore,

Table 4.1.-Information on 24 wolf packs sampled over a 12 year period.

Pack Name[a]	N[b]	Number of Males	Number of Females	Span of years during which radio-collared animals were present
Jackpine	12	7	5	1984-2000
Basin	11	6	5	1985-1995
Jocko	11	6	5	1985-1999
Travers	10	6	4	1983-2000
Limestone[c]	6	3	3	1991-2000
Pretty	7	3	4	1989-1997
Foys[c]	6	2	4	1984-1992
Mathews[c]	6	4	2	1986-1998
MacDonald	5	2	3	1992-1999
Redpole	5	3	2	1980-1999
Acorn	4	1	3	1993-1999
Byers	3	1	2	1991-1998
Northeast	2	2	0	1989-1999
Annie Bay	2	0	2	1985-2000
Eastgate	2	1	1	1985-1989
Grand	2	1	1	1988-1993
Black Bay	1	1	0	1995-1996
Cybulski	1	1	0	1993-1996
Hardwood	1	1	0	1993-1998
Killaloe	1	0	1	1992-1995
Lavielle	1	0	1	1988-1990
Military	1	1	0	1993-1998
North Bissett	1	0	1	1993-1998
Poplar	1	0	1	1997-1999
Average/Pack	4.25	2.17	2.08	

[a] see Figure 1b for pack locations.
[b] N is the number of individuals sampled within the pack.
[c] The 5 pups sampled were not radio-collared, therefore their life span is undetermined and not included in the span of years column.

Table 4.2.- Number of radio-collared animals in packs by year.

Pack	1987		1988		1989		1990		1991		1992		1993		1994		1995		1996		1997		1998		1999	
	M[a]	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Basin	1	1	1	1	2	0	5	0	5	1	6[b]	2	5	3	4	3	0	3	0	0	0	0	0	0	0	0
Jocko	1	0	1	0	2	1	3	1	3	1	3	1	3	1	4	1	3	2	3	4	2	4	2	3	1	1
Jackpine	1	2	2	2	2	2	1	2	1	2	1	3	1	4	1	4	1	5	0	3	1	3	3	2	3	2
Travers	1	0	2	0	4	1	4	1	4	1	3	2	5	2	2	2	2	2	2	1	2	1	0	2	0	2
Pretty	0	0	0	0	0	1	2	1	2	2	2	3	3	2	2	3	2	2	2	1	1	0	0	0	0	0
Limestone	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	1	1	2	1	2	2	2	2	2	2
Foys	2	1	2	1	2	1	2	4	2	4	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mathews	0	1	0	1	1	1	1	1	1	1	2	2	3	2	2	2	2	2	1	1	1	1	0	1	0	0
MacDonald	0	0	0	0	0	0	0	0	0	0	1	0	1	2	2	2	2	3	2	3	1	3	1	1	0	1
Redpole	1	0	1	0	1	0	1	0	2	0	3	1	3	2	3	2	2	0	1	0	1	0	1	0	1	0
Acorn	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3	1	3	1	3	1	3	0	3	0	1
Byers	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	2	1	2	1	2	1	1	1	1	0	0
Total / Year	8	6	10	8	15	11	20	13	24	16	25	21	32	24	28	30	22	29	19	23	18	21	12	19	8	11

[a] Numbers of males (M) and females (F) represent radio-collared animals that were alive for each year in a given pack.

[b] Highlighted years indicate periods of the 12-year study in which the largest number of individuals were alive at the same time for each corresponding pack.

the 102 samples collected represent approximately 22% of the individuals present in the

24 packs over that time period.

Only 12 of these 24 packs had three or more members sampled (Table 4.1). These

packs were used to assess maternity, paternity and kin relationships. The number of

individuals sampled in each of the packs fluctuated considerably over the twelve years

(Table 4.2). The period during the 12-year study, in which the largest number of

individuals was alive in each pack, was between 1993 and 1997. As packs dissolved

others formed or expanded their territory. For example as the Foys pack (Table 4.2) (Fig.

4.1a) began to dissolve in the early 1990s, the existing Basin, Jocko and Redpole packs

extended their territories into part of the Foys area and the new MacDonald pack formed.

Relatedness of individuals in packs

To test the hypothesis that packs primarily comprised an unrelated pair and their

offspring we established DNA profiles at 17 autosomal microsatellite loci, 4 Y

chromosome microsatellite loci and the mitochondrial control region. Eight

mitochondrial haplotypes (C1, C9, C13, C14, C16, C17, C19 and C22) (Table 4.3) were

found in the park. One (C22) was of gray wolf origin and was found in only 4 full

siblings, two were eastern wolf origin and 6 were of western coyote origin. Seven Y

chromosome haplotypes (AA, BB, CC, CD, CE, DC and EF) were identified (Table 4.4).

The 2 frequent haplotypes AA and BB appeared to be of eastern timber wolf origin and

none appeared to be of western coyote origin (Karmi et al., pers. comm.). The frequency

Table 4.3.- Mitochondrial control region haplotypes in Algonquin Provincial Park and surrounding animals.

Haplotype	Algonquin Provincial Park[a]	Frontenac Axis	Magnetawan Region	Northeastern Ontario	Abitibi-Temiscamingue region (Quebec)	La Verendrye Reserve (Quebec)
C1	12	15	1	1	5	1
C3	0	0	0	0	1	0
C9	18	31	13	2	0	0
C13	5	0	2	4	1	0
C14	35	11	6	9	2	1
C16	1	0	0	0	2	2
C17	9	1	0	0	1	0
C19	18	16	4	0	1	0
C22	4	0	0	16	0	9
C36	0	0	0	1	0	0
Total	102	74	26	33	13	13

[a] Two Algonquin park animals could not be profiled at the mitochondrial control region.

Table 4.4- Y chromosome variation in Algonquin Provincial Park animals and surrounding populations.

Haplotype	Algonquin Provincial Park	Frontenac Axis	Magnetawan Region	Northeastern Ontario	Abitibi-Temiscamingue region (Quebec)	La Verendrye Reserve
AA	30	13	5	1	1	0
AF	0	0	0	1	0	0
BB	13	0	2	2	2	0
CC	2	0	0	1	1	1
CD	4	6	1	0	0	0
CE	1	0	1	1	1	1
CF	0	1	0	0	0	3
CI	0	1	0	0	0	0
CS	0	0	0	0	2	1
CT	0	0	0	1	0	0
DC	1	0	0	0	0	0
EF	1	0	0	0	0	0
FL	0	1	0	1	0	0
GP	0	3	0	0	0	0
HS	0	1	0	0	0	0
HT	0	1	0	1	0	0
Total Males	52 (50%)[a]	27 (36%)	9 (35%)	9 (27%)	7 (54%)	6(46%)

[a] Percentages in parentheses indicates proportion of males in population.

of each haplotype in the park population varied from 1% (C16) to 34% (C14) for

mitochondria and 1% (CE, DC, EF) to 58% (AA) for Y-chromosomes. The number of

alleles present at the autosomal microsatellite loci in the park ranged from 4 at locus

cxx.204 to 25 at locus c.2202. An example of the application of the DNA profiles to

examine the relatedness of individuals in a pack is shown in Fig 4.2. The alpha male

breeder is confirmed as it shares one allele at each of the 17 loci with the four offspring as

well as Y chromosome haplotype, AA, with the male offspring. The mother's genotype

(individual not sampled) can be inferred for each offspring and one mother is consistent

for all 4 siblings. This is supported by the mitochondrial haplotype C22 (Roy et al.

1996), which is found in all four offspring and confirmed by the high relatedness values

among the four siblings (p < 0.001), which are consistent with full-sib relationships. In

1996 when this father and his four offspring were present, 2 additional yearlings or sub-

adults were present (Table 4.5) (Fig. 4.4). The male had a mitochondrial haplotype, C17

(9%), which was different from the offspring, but the Y-haplotype (AA) was the same as

the alpha male. The female had a mitochondrial haplotype, C14 (34%), which was

different from both the offspring and the sub-adult male. The alpha male and sub-adult

male appear unrelated based on the kinship analysis (p < 0.05). The 2 subordinate animals

therefore appear to have joined the pack through adoption or pack fusion.

In 1992 the Basin pack had 8 individuals sampled (Table 4.5) (Fig. 4.4), 6 males

and 2 females. One putative father-daughter relationship was identified. The putative

father had a C14 (34%) mitochondrial haplotype and the AA Y-haplotype (58%), while

the female yearling had a C19 (Wilson et al. 2000) mitochondrial haplotype. In addition

Table 4.5.- Mitochondrial DNA and Y chromosome haplotypes found in packs in which a parent offspring relationship was identified.

Packs[a]	MALES			FEMALES	
	Y Haplotype	MtDNA Haplotype	# of individuals	MtDNA Haplotype	# of individuals
Basin (n=8)	AA	C1	2	C1	1
1992	AA	C9	1	C14	1
	AA	C14	2	C19	1
Jocko (n=7)	AA	C14	1	C14	1
1996	AA	C17	1	C22	3
	AA	C22	1	-------	-------
Jackpine (n=6)	BB	C9	1	C9	1
1995	-------	-------	-------	C14	4
Limestone (n=6)	EF	C14	1	C13	3
1997	CD	C13	1	-------	-------
	CD	C19	1	-------	-------
Foys (n=6)	AA	C1	1	C1	2
1990	AA	C14	1	C14	1
	-------	-------	-------	C19	1
MacDonald (n=5) 1995	AA	C14	1	C14	2
	BB	C9	1	C19	1
Redpole (n=4)	BB	C1	1	C19	2
1993	BB	C19	2	-------	-------
Byers (n=3)	BB	C14	1	C14	1
1993	-------	-------	-------	C17	1

[a] Information of each pack is based on the year over the 12 year study in which the greatest number of individuals were alive.

Fig. 4.4.- Three packs identified with at least one putative parent offspring relationship.

A. The Jocko Pack (1996) B. The Basin Pack (1992) C. The Limestone Pack (1997).

Putative parents (closed black); Parents not sampled (closed gray); Putative offspring

unrelated (black dots); Full-sibs (black stripes); Half-sibs (black diamonds); Unrelated

subordinates (open). Name of animal is followed by a mitochondrial haplotype / Y

haplotype. Mitochondrial haplotypes are designated with a capital C followed by a

number.

A. Jocko Pack (1996)

Jocko 10
C14 / AA

Jocko 9
C22

Jocko 12
C22

Jocko 13
C22

Jocko 11
C22 / AA

Subordinate Female

Jocko 7 C14

Subordinate Male

Jocko 8 C17/ AA

B. Basin Pack (1992)

Basin 4
C14 / AA

Basin 9 Macdonald
C19

Subordinate Female

Basin 10 C1

Subordinate Male

Basin 6 C14/ AA

Basin 7 C14/ AA

Basin 8b C9/ AA

Basin 12 C1/ AA

Basin 14 C1/ AA

C. Limestone Pack (1997)

Limestone 2
C13

Limestone 4
C19 /CD

Limestone pup3
C13

Limestone pup1
C13 / CD

Limestone pup2
C13

Subordinate Male

Limestone 3 C14 / EF

the pack contained three adults (> 2 years of age) and three pups or yearlings. The 2 male adults had the C14 mitochondrial haplotype and the AA Y-haplotype. Relatedness values indicate that these two males are full-sibs (R = 0.647). The single adult female had a mitochondrial haplotype C9 (Wilson et al. 2000), which differed from that of the adult males and putative offspring. The remaining individuals were either pups or yearlings in 1992. All three pups / yearlings had the same mitochondrial haplotype, C1 (12%) and both males had the common AA Y-haplotype. All 3 yearlings have relatedness values indicative of a full sib relationship. Therefore again this pack was not simply a breeding pair and their offspring.

In the Limestone pack, located outside the southern borders of Algonquin Park (Fig. 4.1a) a putative mother and her three offspring (2 females, one male) were identified during 1997-1998. For two of these offspring the putative father was also identified (Fig. 4.2). The putative mother had a C13 mitochondrial haplotype (5%) (Table 4.5) and the putative father had a C19 mitochondrial haplotype in addition to a CD Y-chromosome haplotype (8%). The mated pair was unrelated (p < 0.05). One additional adult male was found in the pack with an EF Y-haplotype (2%) and a C14 (34%) mitochondrial haplotype. This subordinate male was not related to the putative alpha male and may possibly have entered into the pack through adoption.

Similar complex situations were found in most of the packs and overall the data suggest that packs in the park are rarely simply an unmated pair and their offspring.

Origin of subordinates in Packs

To assess the origin of the non-breeding subordinates we first focused on the Foys

pack, which dissolved during the study. Three packs expanded their territory onto the

Foys territory in 1992, the Basin pack, the Jocko pack and the Redpole pack. In the same

year the MacDonald pack formed in part of the territory (Fig. 4.1a). We assessed the pack

origin of the subordinate animals. With the exception of the Redpole Pack all subordinate

and alphas within the packs had the same common Y chromosome haplotype AA (58%)

(Table 4.6). A number of relationships were identified amongst these males all of which

had R-values greater than 0.260 (Table 4.6). Many of the relatedness values indicate the

presence of a number of second- degree relatives between various members of all 5

packs. Within the Redpole pack all 3 males appear to be related as half sibs or full sibs.

Based on demographic data and the high relatedness values, it is possible that Redpole 4

is the grandfather of full sibs Redpole 3 and Redpole 5. Amongst the remaining related

dyads 5 have relatedness values of >0.500 indicating a first degree relationship. Two

were previously identified as full sibs within their respective packs. The remaining dyads

are Jocko 2 and Basin 12, Jocko 2 and Basin 14 and Redpole 4 and Basin 12 (Table

4.5a). In contrast, only one related dyad was observed amongst the females of this pack

system. Three different mitochondrial haplotypes C1 (12%), C14 (34%) and C19 (Wilson

et al. 2000) were identified among the subordinate females (Table 4.6). The C1

haplotype was identified in two subordinate females with a relatedness value indicative of

an unrelated pair. The C14 haplotype was present in another three subordinate females in

which one of the dyads (Redpole 2 and Foys 6) had a relatedness value (0.255) indicative

Table 4.6.- Alpha males and subordinate males within a system involving 5 packs in 1992.

Males	Y-Haplotye	Mitochondrial Haplotype	Relationship[a]	Relatedness Values
Basin 4	AA	C14	Basin 7	0.298
			Jocko 6	0.260
Basin 6	AA	C14	Basin 7	0.647
Basin 7	AA	C14	Jocko 6	0.281
Basin 8b	AA	C9		
Basin 12	AA	C1	Basin 14	0.560
			Jocko 2	0.640
			Jocko 6	0.306
			Redpole 3	0.338
			Redpole 4	0.538
Basin 14	AA	C1	Jocko 2	0.538
			Redpole 4	0.444
			Redpole 5	0.282
Jocko 2	AA	C1	Jocko 6	0.329
			Redpole 4	0.393
			Redpole 5	0.328
Jocko 4	AA	C17		
Jocko 6	AA	C14		
MacDonald 9	AA	C14		
Redpole 3	BB	C19	Redpole 4	0.384
			Redpole 5	0.496
Redpole 4	BB	C1	Redpole 5	0.474
Redpole 5	BB	C19		

[a] shaded areas represent the absence of a relationship with individual male from column one.

of a second-degree relationship. Only 1 female subordinate had a C19 mitochondrial haplotype (Table 4.7) and she was not related to any of the other subordinate females.

With the exception of a possible half-sib relationship between the one female dyad with the C14 mitochondrial haplotype, the female subordinates in these adjoining packs do not appear related. In contrast the male breeders and subordinates within this pack system are related. The data suggest that subordinates in adjoining packs or in a pack system are related males that remain in close proximity to each other even after pack splitting and pack fusion.

To further identify sources of unrelated subordinates in a pack we looked at two periphery packs, each with a large number of unrelated individuals. In 1994, the Pretty pack, located on the northeast periphery of Algonquin Provincial Park had 5 individuals sampled (Table 4.8), 2 males (one adult and one pup/yearling) and 3 females (two adults and one pup/yearling). Although no putative parent offspring relationships were identified within the Pretty pack, two putative parent offspring relationships were identified with members of surrounding packs. In 1990 a female from the Grand pack gave birth to a male (C19 mitochondrial haplotype and a AA Y-haplotype) and in 1992 a male from the Jocko mated with an unknown female to produce a female (C14 mitochondrial haplotype). In 1994 both offspring were present in the Pretty pack as adult members. No relationship between them and other current Pretty pack members have been identified. The remaining adult in the pack was a female with a mitochondrial haplotype (C19) and unrelated to other members within the pack. In addition to the adults 2 pups or yearlings were present and both had the same mitochondrial haploype (C9),

Table 4.7.- Alpha females and subordinate females within a system involving 5 packs in 1992.

Females	Mitochondrial Haplotype	Relationship[a]	Relatedness Value
Jocko 3	C1		
Basin 10	C1		
Basin 9	C19		
Redpole 2	C19	Foys 6	0.255
Foys 6	C19		
Foys 7	C14		

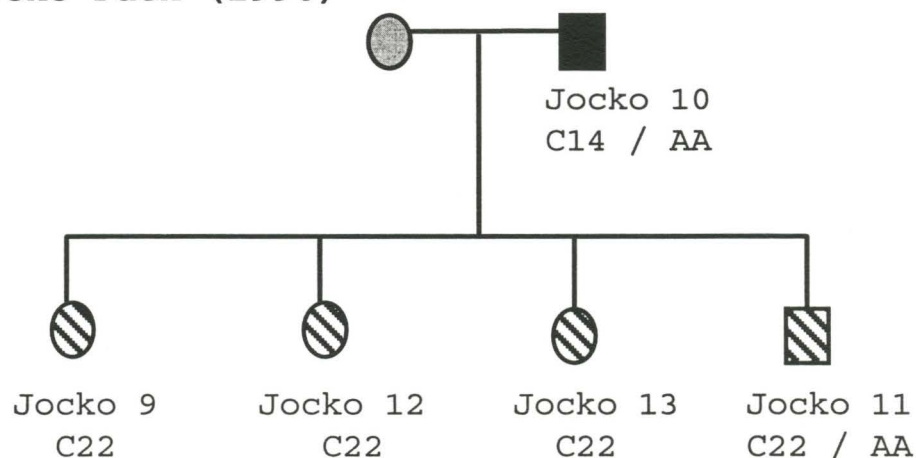[a] shaded areas represent the absence of a relationship with individual male from column one.

Table 4.8.- Mitochondrial DNA and Y chromosome haplotypes found in packs with no parent offspring relationships identified.

Packs[a]	MALES			FEMALES	
	Y Haplotype	MtDNA Haplotype	# of individuals	MtDNA Haplotype	# of individuals
Travers (n=7)	AA	C14	2	C14	2
1993	BB	C1	1	-------	-------
	DC	C9	1	-------	-------
Pretty (n=5)	AA	C9	1	C9	1
1993	AA	C17	1	C14	1
	AA	C19	1	-------	-------
Mathews (n=5)	AA	C17	1	C9	1
1993	AA	C19	1	C14	1
	BB	C9	1	-------	-------
Acorn (n=4)	AA	C14	1	C9	1
1994	-------	-------	-------	C14	1
	-------	-------	-------	C19	1

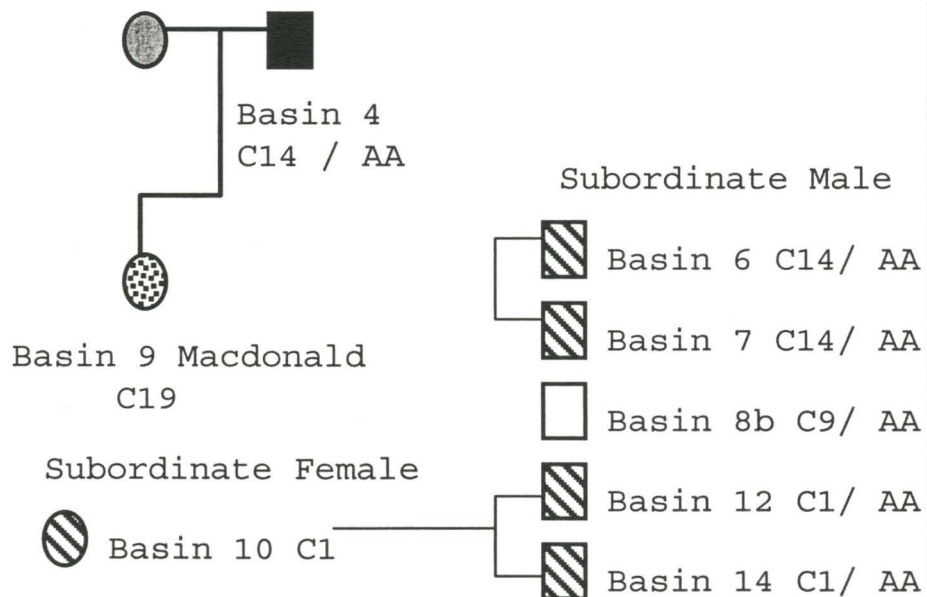[a] Information of each pack is based on the year over the 12 year study in which the greatest number of individuals were alive.

which was different from all 3 adults. The female pup or yearling was identified as an immigrant into the Park (Table 4. 9) using the program STRUCTURE. The formation of the Pretty pack appears to be complex with subordinates coming from surrounding Park packs as well as immigrants into the Park.

The Travers pack is also located on the northern periphery of Algonquin Park and in 1993 had 7 members present (Table 4.8), 5 males and 2 females. No putative parent offspring relationships were identified between members within this pack or between it and surrounding Park packs. Of the 7 members, four were adults (three males and one female) and 3 were pups or yearlings. All 3 adult males have different mitochondrial haplotypes C17 (9%), C1 (12%) and C9 (Wilson et al. 2000) and different Y-chromosome haplotypes, AA (58%), BB (25%) and DC (2%) respectively. Two of these males, 1 with the common AA Y haplotype and the other with a DC Y-haplotype were identified as immigrants into the Park (Table 4.9) using the program STRUCTURE. The adult female present in the pack had a C17 mitochondrial haplotype. The remaining three animals were yearlings or pups in 1993 (two males and one female). All yearlings had the same mitochondrial haplotype, C14 (34%), which is different from that of the 4 adults. The absence of any related pairs and the presence of four mitochondrial haplotypes and two Y haplotypes in the Travers pack (Table 4.8) suggests the Travers pack was formed either from the fusion of other packs or several independent adoption and immigration events.

The origin of subordinates appears to arise from a complex mixture of events including dispersal, immigration, pack fusion and adoption.

Table 4.9.- Assessment of Immigrants into Algonquin Park.

Animals	Population Origin	MtDNA Haplotype	Y-Haplotype	Pack Position in Park
Travers 2	Frontenac	C9	AA	north
Pretty 8[a]	Frontenac	C9	-----	north
Jackpine 4[a]	Frontenac	C1	CD	central
Killaloe	Magnetawan	C1	-----	south
Travers 5	Northeastern Ontario	C9	DC	north

[a] These animals represent 2 of the 5 smallest park animals sampled.

Genetic Relationship of a pack over generations

The presence of western coyote mtDNA haplotypes and the absence of western coyote Y-chromosome haplotypes in the park wolves suggest asymmetric mating between eastern timber wolves and western coyotes. It has been suggested this may be as a result of female choice (Karmi et al., pers. comm.). We wished to examine whether territories were largely passed from father to son or mother to daughter. We therefore assessed whether Y-chromosome or mitochondrial haplotypes were more persistent in a pack through generations.

Six packs in the study had at least one member sampled for at least 10 of the 12 years. Three of the packs had a single persistent Y-haplotype over the generations (Table 4.10). The Jocko (6 males and 5 females) and Pretty (3 males and 4 females) packs had the common AA Y-haplotype, whereas the Redpole pack (3males and 1 female) had the BB Y-haplotype (Table 4.10). Among the females, 3 mitochondrial haplotypes (C1, C14 and C22) were found in the Jocko pack, two in the Pretty pack (C9 and C14) and a single haplotype in the Redpole Pack.

The remaining packs had multiple Y-chromosome and mitochondrial haplotypes. The Mathews pack had 6 individuals sampled (4 males and 2 females) (Table 4.10). Two Y-chromosome haplotypes, AA and BB were identified in the males and 2 mitochondrial haplotypes, C9 and C14 were identified in the females. The remaining Jackpine and Travers packs each had three Y haplotypes present in the males and 2 mitochondrial haplotypes in the females. Males in both packs had the AA and BB Y-haplotypes. The third Y-haplotypes identified in the Jackpine and Travers packs were rare, CD (8%) and

Table 4.10.- Mitochondrial DNA and Y chromosome haplotypes found in packs with at least one individual sampled in a given year over the 12 years of study.

Packs	MALES Y Haplotype	MtDNA Haplotype	# of individuals	FEMALES MtDNA Haplotype	# of individuals
Jocko (n=11)	AA	C1	1	C1	1
	AA	C14	2	C14	1
	AA	C17	2	C22	3
	AA	C22	1	-------	-------
Jackpine (n=11)	AA	C14	3	C9	1
	AA	C19	1	C14	4
	BB	C9	1	-------	-------
	CD	C1	1	-------	-------
Travers (n=10)	AA	C14	2	C9	1
	AA	C9	1	C14	3
	BB	C1	2	-------	-------
	DC	C9	1	-------	-------
Pretty (n=7)	AA	C9	1	C9	3
	AA	C17	1	C14	1
	AA	C19	1	-------	-------
Mathews (n=6)	AA	C9	1	C9	1
	AA	C17	1	C14	1
	AA	C19	1	-------	-------
	BB	C9	1	-------	-------
Redpole (n=4)	BB	C1	1	C19	2
	BB	C19	2	-------	-------

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DC (2%) respectively. These males were identified as immigrants into the Park (Table 4.10) using the program STRUCTURE. Two mitochondrial haplotypes were identified amongst the females in both packs.

The Y-haplotype appears to be persistent more often in the packs than the mitochondrial DNA. However the persistent Y haplotypes are most often the common AA haplotype, which occurs in 58% of the population. With the complex formation of these Park packs, maternal and paternal lineages within packs even over long generations are difficult to assess.

<div align="center">Identification of Immigrants</div>

One potential threat to the Algonquin Park wolf packs that has been recognized is gene swamping from coyote genetic material as a result of hybridization with the eastern coyotes or "Tweed" wolves found in the Magnetawan and Frontenac Axis. Related to this is the question of whether the park wolves represent an island population with a small effective population size or whether they are part of a larger metapopulation of *C. lycaon*. One way to examine this is to determine the origin of the animals in the park. Using the program STRUCTURE we identified 5 immigrants into the park. This is a conservative minimum estimate, as only animals from distinctly different populations will be identified. Three appeared to originate from the Frontenac Axis, one from the Magnetawan region. These had genotypes that showed more coyote genetic material One appeared to originate from north of the park and showed more gray wolf genetic material (Table 4.6). Two of the coyote impacted animals were within the 5 smallest animals

recorded in the park. There is no evidence that any immigrants reproduced in their respective packs.

Genetic Relationship of Park animals to those in surrounding areas

The majority of the mitochodrial and Y-chromosome haplotypes in the park are present in the surrounding regions. (Tables 4.3 and 4.4). Using ϕ_{ST} in the program AMOVA (Analysis of Molecular Variance, version 1.55; Excoffier et al. 1992) structuring among the regions was identified (Table 4.11). The mitochondrial and Y-haplotypes among Northeastern Ontario, the Abitibi-Temiscamingue region and the La Verendrye appear to be less structured with each other than to Algonquin Park, the Magnetawan region and the Frontenac Axis. This is consistent with more coyote genetic material in the Algonquin Park animals and more gray wolf genetic material in the animals to the north. We calculated both R_{ST} and F_{ST} using the data at the 8-microsatellite loci (Table 4.12). Similar relative values were found with R_{ST} and F_{ST}, therefore, since R_{ST} takes into account the step-wise mutation model, which is associated with microsatellites we used R_{ST} values for structuring comparisons. With the exception of the Magnetawan region, the Frontenac Axis animals show limited gene flow with all animals in other areas including Algonquin Provincial Park and those in Quebec. Lower levels of structuring are observed across the western and northern borders of Algonquin Park between Northeastern Ontario and its surroundings, which include both the Abitibi-Temiscamingue region and the La Verendrye reserve as well as the Magnetawan region and Algonquin Provincial Park.

The apparent gene flow between Algonquin Park and the surrounding western and

Table 4.11. φ_{ST} values for Y haplotype (above diagonal) and mitochondrial haplotype (below diagonal) for each pairwise comparison of Algonquin Park and surrounding regions.

Population	Frontenac Axis	Algonquin Provincial Park	Magnetawan Region	Northeastern Ontario	Abitibi-Temiscamingue region (Quebec)	La Verendrye Reserve (Quebec)
Frontenac Axis	X	0.0568	-0.0054	0.1114	0.1416	0.2321
Algonquin Provincial Park	0.0594	X	-0.0578	0.1331	0.1356	0.3250
Magnetawan Region	0.0090	0.0583	X	0.0493	0.0545	0.2441
Northeastern Ontario	0.2354	0.1334	0.2282	X	-0.0569	0.0733
Abitibi-Temiscamingue region (Quebec)	0.1191	0.0623	0.1826	0.1900	X	0.0562
La Verendrye Reserve (Quebec)	0.3292	0.2536	0.3651	0.0424	0.2664	X

Table 4.12.-R_{ST} (above diagonal) and F_{ST} (below diagonal) values for each pairwise comparison of Algonquin Park and surrounding regions.

Population	Frontenac Axis	Algonquin Provincial Park	Magnetawan Region	Northeastern Ontario	Abitibi-Temiscamingue region (Quebec)	La Verendrye Reserve (Quebec)
Frontenac Axis	X	0.114	0.040	0.102	0.161	0.190
Algonquin Provincial Park	0.055	X	0.057	0.036	0.070	0.067
Magnetawan Region	0.024	0.021	X	0.016	0.047	0.066
Northeastern Ontario	0.076	0.072	0.052	X	0.025	0.018
Abitibi-Temiscamingue region (Quebec)	0.089	0.049	0.041	0.030	X	-0.027
La Verendrye Reserve (Quebec)	0.091	0.051	0.043	0.012	0.017	X

northern populations suggest that Algonquin Park is not an island population, but part of a connected metapopulation. These data are supported by the low inbreeding (F_{IS}) and high levels of both allelic diversity (A) and observed heterozygosity (H_O) within the Park (Table 4.13). Using 8 of the 17 autosomal loci, allelic diversity and heterozygosity values in Algonquin Provincial Park animals are 6.6 and 0.7 respectively. This compares to average allelic diversity (7.3 and 6.3) and heterozygosity values (0.7 and 0.7) for gray wolf and western coyote populations (Table 4.13). Hybridizing eastern timber wolf populations averaged 6.3 and 0.7 respectively. The captive red wolf, which would be expected to have the least amount of variation, had diversity levels of 3.6 and 0.5 respectively. Allelic diversity levels between populations used in this study and those of gray wolves and western coyotes are similar (Table 4.13).

DISCUSSION

The traditional view of a pack of wolves is that it is composed of a dominant breeding pair (alpha male and female) and their offspring (Mech 1970, 1987). The subordinate or beta animals were thought to be the offspring of the alpha pair from the previous season (Murie 1944; Mech 1970). Occasionally animals were seen to join a pack (adoption / pack mergers) (Fritts and Mech 1981; Van Ballenberge 1983; Fuller 1989) and new packs formed either by wolves dispersing from a nearby pack (Fritts and Mech 1981) or from the splitting/budding of existing packs (Jordan et al. 1967; Mech 1986; Meier 1995). The 12 years of study of the packs of wolves in Algonquin Provincial Park and the detailed DNA profiles of over 100 of these animals allowed us to examine this

Table 4.13.- Information on North American wolf and coyote populations.

Populations	Habitat	N^a	# of loci	A^b	heterozygosity	F_{IS}
Non-hybridizing Gray Wolf[c]	natural	16.8	10	6.4	0.741	N/A
Northwest Territories	natural	125.5	8	7.8	0.691	N/A
Pukaskwa National Park	natural	13.0	8	4.8	0.682	N/A
Average (gray wolf)				7.3	0.655	N/A
Algonquin Provincial Park	natural	84.5	8	6.6	0.650	0.001
Red Wolf	captive	60.0	8	3.6	0.493	N/A
Hybridizing Populations				5.2	0.572	
Frontenac Axis	natural	74.0	8	7.6	0.712	0.039
Magnetawan Region	natural	25.8	8	6.5	0.702	0.036
Northeastern Ontario	natural	33.0	8	6.9	0.732	0.017
Abitibi-Temiscamingue region (Quebec)	natural	13.0	8	5.4	0.677	-0.038
La Verendrye Reserve (Quebec)	natural	13.0	8	5.0	0.690	-0.097
Red Wolf	wild	129.0	8	6.6	0.486	N/A
Average (lycaon hybrid variants)				6.3	0.667	N/A
Non-Hybridizing Coyotes[1]	natural	17	10	5.9	0.675	N/A ·
Texas	natural	23.8	8	6.0	0.678	N/A
NorthCarolina	Natural	23	8	7.0	0.759	N/A
Average (coyote)				6.3	0.704	N/A

[a] N represents the number of individuals analysed.

[b] A represents the allelic diversity.

[c] Roy et al. (1994).

traditional view and test the hypothesis "a pack of eastern timber wolves is composed of an unrelated pair of breeding adults (alpha male and alpha female) and their offspring" (Mech 1970). Surprisingly many subordinates, pups and yearlings were found to be unrelated to the breeding pair. The hypothesis is therefore rejected and the data are consistent with the suggestion that many pack members often originate through pack adoptions or fusions and through dispersal and pack splitting/ budding. Studies on the eastern timber wolves of Minnesota (Fritts and Mech, 1981; Mech and Nelson 1990; Lehman et al. 1992) are consistent with this more complex view of a pack. Lehman et al. (1992) used genetic markers to compare packs of eastern timber wolves in Minnesota with two gray wolf populations from Alaska and the Northwest Territories. There were no major differences suggesting the packs of both gray and eastern timber wolves are not as simple as the traditional view.

A possible explanation of the complex pack structures identified in Algonquin Park is the high mortality observed when animals leave the Park (Forbes and Theberge 1995). This mortality is primarily a consequence of long-range excursions by wolves outside park boundaries in search of deer during the winter. Seasonal migrations or extraterritorial movements by wolves have been reported in a number of studies (Parker 1973; Carbyn 1981; Van Ballenberghe 1983; Peterson et al. 1984; Messier 1985), however their consequences on pack structure is poorly understood. In populations where harvesting and human related mortality is high (Rausch 1967; Van Ballenberghe et al. 1975), increased recruitment and acceptance of wolves into packs has been observed (Van Ballenberghe 1983). Adoptions have also been identified in unharvested

populations such as Denali, where wolves were accepted into long-established packs as well as in new packs with a single generation of pups (Meier et al. 1995). It is difficult to determine whether the complex pack structures observed are normal or a result of these factors, as there are insufficient studies on packs with varying levels of mortality and migration (Harber, 1996). As the packs of eastern timber wolves and gray wolves studied in Minnesota, Alaska and the Northwest Territories show similar complex relationships within packs this may be the norm rather than a consequence of higher mortality and migration.

In this study we focus on the hypothesis that as the eastern timber wolf moved north into Algonquin Park at the end of the 19th century it largely encountered an environment in which the gray wolf had been severely reduced. The eastern timber wolf would have flourished in the Park since white-tailed deer had also moved north and would have been abundant. Around 1930-1950 the eastern coyotes that originated from south- western Ontario would have reached the area. The wolf control programs of the Algonquin Park rangers continued into the 1960s, which probably promoted hybridization of the eastern timber wolves in the Park with the eastern coyotes. A high proportion of samples of Algonquin wolves from the 1960's contained western coyote mtDNA haplotypes but no gray wolf haplotype were found (Wilson et al. 2000). Today most of the mitochondrial haplotypes are of eastern timber wolf or western coyote origin, with the exception of four full sibs, which have a gray wolf mitochondrial haplotype. These may have originated from the original Park inhabitants or entered as a result of gene flow from the animals to the north of the Park.

In contrast to the presence of western coyote mitochondrial DNA, there are no western coyote Y-chromosome haplotypes and most males contain Y-chromosomes originating from eastern timber wolves (Karmi et al., in prep.). Some males contain Y-chromosomes of gray wolf origin and these may have come from the original Park inhabitants or gene flow from the north. The presence of western coyote mtDNA haplotypes and the absence of western coyote Y-chromosome haplotypes in the park wolves suggest asymmetric mating between eastern timber wolves and western coyotes. It has been suggested this may have resulted from more widely dispersing females or mate choice of females (Karmi et al., in prep.). If correct, we would predict a persistence of Y haploytypes within packs or pack systems. We tested whether territories were more often passed from mother to daughter than father to son based on the persistence of Y-chromosome or mtDNA haplotypes through generations. The data do allow rejection of matrilineal transmission and is generally consistent with the idea of patrilineal inheritance of a territory. The persistence of a small number Y haplotypes in the park coincides with the Y stickiness or patrilocality observed in North American wolves and coyotes (Karmi et al., in prep.). Y-haplotypes are persistent in a number of packs studied over the 12 years. As well a large number of relationships between alpha males and subordinates were identified amongst 5 adjacent packs.

One of the threats to the long-term persistence of wolves in Algonquin Park is gene swamping by western coyote genetic material. By the 1960s there was evidence of hybridization between the eastern timber wolves of the Park and the eastern coyotes. The animals in the Park are clearly different from those outside in areas such as the Frontenac

Axis (Sears 1999). The genetic structuring data shows limited gene flow from the "Tweed wolves" of the southeast into Algonquin Provincial Park. From this region three immigrants into the Park were identified. Immigrants are identified when two populations are different. In genetically similar populations, identification of immigrants would be more difficult. None of the immigrants from the Frontenac were involved in a parent offspring relationship. Overall the data do not support the suggestion that coyote introgression is a potential threat to the integrity of the eastern timber wolf in Algonquin Provincial Park. It seem likely that most of the western coyote mitochondrial DNA haplotypes entered the Park following wolf culls that occurred up to the 1960s and when smaller "Tweed" wolves may have flourished in a region with abundant white-tailed deer. Given a low deer density in the Park it is likely that smaller animals are selected against and this is the basis of the present restricted gene flow.

The limited gene flow from the southeast leads to the question of whether the Algonquin Park wolves are an island population of a few hundred animals or connected to a larger meta-population. The Rst data support the model that Algonquin Park animals are the southern group of animals of a larger metapopulation. There is gene flow with animals to the north of Algonquin Park between northeastern Ontario and Quebec's Temiscamingue-Abitibi region and the La Verendrye reserve. Only one immigrant was identified from northeastern Ontario into the park. The connectivity with the north is supported by the lack of inbreeding and moderately high levels of heterozygosity and allelic diversity identified within Algonquin Provincial Park. Diversity levels are similar to those of gray wolf and western coyote populations (Roy et al. 1996). This variation

coupled with the high gene flow among groups of animals to the east and north, suggests that the Park animals are not an island population, but the southern part of a larger metapopulation of *C. lycaon*, that stretches from Quebec to Manitoba and includes the animals in Minnesota, Wyoming and Michigan.

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Preface

This chapter was written with co-authors and formatted for submission to a peer-reviewed journal and is therefore a self contained manuscript with an individual reference section.

Chapter 5: Allelic variation at the DLA DQA locus of the Major Histocompatibility

Complex (MHC) in North American wolves and coyotes

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Status: Submitted to Heredity for review.

Contribution: Major histocompatibility complex data collection done by S.K.G. and

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was conducted under the supervision and guidance of B.N.W.

Chapter 5

Allelic variation at the DLA DQA locus of the Major Histocompatibility Complex (MHC) in North American wolves and coyotes

ABSTRACT

We examined the allelic variation of the major histocompatibility complex (MHC) class II DQA locus in wolves and coyotes in North America. The four species involved in this study were gray wolves, (*Canis lupus*) from the Northwest Territories, the eastern timber wolf, (*C. lycaon*) from Algonquin Provincial Park, the red wolf, (*C. rufus*) from the captive breeding program and western coyotes, (*C. latrans*) from Texas. Cloning, sequencing and single strand conformation polymorphism (SSCP) analyses were used to assess a 246-bp segment of the exon 2 region of the DLA-DQA1 locus for 71 animals. Twelve alleles were identified, seven of which were previously characterized in various breeds of the domestic dog. Many of the alleles were shared among the four species, with the exception of 2 new alleles confined to the Texas coyote population and one allele identified in a dog breed that was found in the Algonquin Park population. Eight of the 9 amino changes found were in the previously identified antigen recognition sites. The number of non-synomous nucleotide substitutions was 3 times higher than the number of synomous changes, indicative of strong positive selection at this locus. These data will provide the basis for the determination of allele frequencies and their distribution across the geographical range of the four species in North America.

INTRODUCTION

The Major Histocompatibility Complex (MHC) genes are members of the immunoglobin superfamily and represent some of the most polymorphic and thoroughly investigated genes in the vertebrate genome (Klein 1986). The genes of the MHC encode cell surface glycoproteins that initiate an immune response by binding foreign antigens (peptides) and presenting them to T-cells. There are currently three recognized classes of MHC molecules – Class I, Class II and Class III. The dog analog of HLA (Human Leukocyte Antigen), called DLA (Dog Leukocyte Antigen) can be divided into three serologically defined loci, DLA-A, DLA-B, DLA-C (Bull et al. 1987), while a fourth region DLA-D is defined by mixed leukocyte culture (Deeg et al. 1986). Molecular analyses (Sarmiento and Storb 1988a, b, Sarmiento et al 1992, Sarmiento et al. 1993, Wagner et al. 1996) have confirmed a number of genes within the DLA-D region. At present four DLA class II alpha genes (DRA, DQA, DPA and DNA) and seven beta genes (2 DRB, 2 DQB, 2 DPB and DOB) have been documented. The degree of polymorphism at the DLA-D region has been studied using various breeds (Sarmiento et al. 1990, Sarmiento et al. 1992; Sarmiento et al. 1993, Wagner et al. 1996, Polvi et al. 1997; Francino et al. 1997; Kennedy et al. 1999; Kennedy et al. 2000). These studies have identified 36 DRB1 alleles, 11 DQA1 alleles and 21 DQB1 alleles. The MHC DLA-DQA locus is one of three genes found in the DLA-DQ region. Recent analyses (Sarmiento et al. 1993; Wagner et al. 1996; Wagner et al. 1998; Kennedy et al. 1999; Kennedy et al. 2000) confirm the presence of one DQA and two DQB genes, only one of which is functional (DQB1). The DLA-DQA allele analysis has shown that most of the

polymorphic sites are within the antigen binding sites which are hypervariable regions comprising 23 amino acids. The acceptance and naming of new DLA alleles is currently controlled by a nomenclature committee working under the International Society for Animal Genetics (Kennedy et al. 1999)

The mechanisms involved in maintaining the high level of polymorphism observed at the MHC genes have been reviewed extensively (Klein and Takahata 1990, Potts and Wakeland 1990, Edwards and Hedrick 1998). One such mechanism involves the pathogen based selection model, which proposes that the high variation of the MHC genes facilitate the recognition of a variety of pathogens and parasites thereby giving an individual a heterozygote advantage (Potts and Wakeland 1990; Potts and Slev 1995; Hill 1998). Associations between MHC genotypes and their alleles with resistance to infectious diseases (Hill et al. 1991; Thursz e al. 1997) as well as parasites (Hughes 1991, Patterson et al. 1998) have been identified. Studies on such associations are however limited and may be in part due to the complex number of genes involved in immune response. A number of MHC and non- MHC genes may be involved and therefore only by building composite haplotypes for a number of genes may direct associations between genotypes and disease be made.

Currently in North America, three species are generally recognized in the genus *Canis*. It has recently been suggested, based on genetic data that the eastern timber wolf is not a subspecies of the gray wolf but a North American-evolved wolf similar to the red wolf, *C. rufus* (Wilson et al. 2000) and it has been proposed it retain its original taxonomic designation of *C. lycaon*. The data suggest that *C. lycaon* and the western

coyote *C. latrans* diverged approximately 150 000 - 300 00 years ago, both evolving

independently from the gray wolf, (*C. lupus*) which evolved in Eurasia 1-2 million years

ago (Wilson et al. 2000). Hybridization between the various species has been identified,

with the exception of *C. lupus* and *C. latrans*. The gray wolf, *C. lupus* used in this study

is the largest of the species preying predominantly on caribou in the Northwest Territories

(Dale et al. 1995). The eastern timber wolf, which extends from Manitoba to the southern

parts of Quebec (Grewal et al. In prep), is a medium size animal preying on white tailed

deer, and beaver in the south (Forbes and Theberge 1995). It preys more on moose in its

northern range and snowshoe hare when deer are at low densities or absent. Texas

coyotes are the smallest and highly adaptable species having expanded throughout much

of North America in the past 100 years. Coyotes consume a variety of smaller prey as

well as berries.

Glaciations, deforestation and control programs have dramatically affected the

distribution of the four species and their interaction in North America. Differences in

habitat and prey base will influence exposure to pathogens and parasites and therefore

selection at the MHC loci. In this study we characterized the allelic variation at the exon

2 region of the class II MHC DLA-DQA locus in wolves and coyotes across North

America.

MATERIALS AND METHODS

Samples

Four groups of samples were used in this study. Northwest Territories gray wolves, *C. lupus* (n=20) Algonquin Provincial Park eastern timber wolves, *C. lycaon* (n=24), captive red wolf samples, *C. rufus*, (n=7) and western coyotes from Texas, *C. latrans* (n=20). DNA was previously extracted using Qiagen (Wilson et al. 2000; Grewal et al. in prep).

PCR amplification

A 246 bp segment of the DLA-DQA exon 2 was amplified by the polymerase chain reaction (PCR) using dog-derived primers from Wagner et al. (1996):

Primer 1: DQA-3A 5' GGA CAG ATT CAG TGA AGA GA 3'

Primer 2: DQA-5A 5' TAA GGT TCT TTT CTC CCT CT 3'

Amplification was carried out with 10 ng of genomic DNA using 200 μM dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$, primers 1 and 2 (0.4 μM), 2.5ug of bovine serum albumin (BSA) (Pharmacia) and 1.0 units of *Taq* polymerase (Gibco BRL). The following thermocycler conditions were used: 94°C for 3 min., 58°C for 1 min., 72°C for 2 min. (3 cycles), followed by 94°C for 15 sec., 58°C for 30 sec., 72°C for 1 min. (27cycles), followed by 72°C for 10 min. PCR products were subjected to electrophoresis in a 1% agarose gel and extracted from the gel using the polyester pillow filling (Mountain Mist brand, Stearns Canada) method.

Cloning of amplified DQα exon 2 fragments

Approximately 100 ng of PCR product were used for ligation into the pGEM-T TA vector (Promega). The PCR product: pGEM-T Vector ligation was transformed into Maximum efficiency DH5α competent cells (Gibco BRL) by incubating at 42°C for 45 seconds. The cells were then incubated at 4°C for 2 minutes, then for 1 hour at 37° C in Luria broth and plated on LB/ampicillin/2% X gal (50 ul)/0.1 M IPTG (100 ul) plates. Using a blue-white screening method, plasmids containing the insert were isolated using the QIAprep miniprep kit (Qiagen Inc.)

DNA Sequencing

Plasmids with DQA exon 2 inserts were sequenced using the ABI 377 DNA automated sequencer (Applied Biosystems). The PCR sequencing reactions were preformed using dye-terminator labeled dideoxynucleotides and the T7 primer. Thermocycler conditions were 96° C for 30 sec., 50° C for 15 sec. and 60° C for 4 min. (25 cycles). Products were purified using the Dye-Ex spin kit (Qiagen). Four clones per sample on average were sequenced.

Single Strand Conformation Polymorphism (SSCP)

Samples were screened for alleles using single strand conformation polymorphism (SSCP). PCR products were electrophoreses through a nondenaturing acrylamide gel (5% acrylamide [59 acrylamide:1 bisacrylamide], 10% glycerol and 0.5X TBE) for 16 hours at

4^{0}C. Prior to loading, products were mixed with 20% formamide, then denatured for 10 minutes (95^{0}C) and placed on ice for 5 minutes. The heterozygotes were sequenced directly and alleles were identified.

Sequence Analysis

DNA sequences were aligned and the amino acid sequence translated using the computer program Bioedit (Hall 1999). Using the program MEGA (Kumar et al. 1993), a neighbour-joining tree was generated using the genetic distances of Jukes and Cantor (1969). Bootstrap values were calculated using 1000 replicates. The sequences were compared to previously published DQA sequences reported in the domestic dog. The analysis of non-synonymous and synonymous substitutions was performed using the program MEGA (Kumar et al. 1993) according to the method of Nei and Gojobori (1986) with the Jukes and Cantor (1969) correction. Alleles previously not identified in the dog were given designations based on the proposed nomenclature (Kennedy et al. 1999).

RESULTS

A total of twelve DLA-DQA alleles were identified among the 71 samples (Fig. 5.1 and 5.2). Seven were previously identified in domestic dogs (Sarmiento et al. 1992, Wagner et al. 1996, Polvi et al. 1997). Four of the original 11 DQA1 alleles identified in domestic dogs were not found in this study. For the five new alleles, all polymorphic sites were within the hypervariable region with the exception of allele DQA1*01202, which

Fig. 5.1. DLA-DQA1 nucleotide sequence alignment.

```
                        10                                              20
DQA1*00101    GAC CAT GTT GCC AAC TAC GGC ATA AAT GTC TAC CAG TCT TAC GGT CCC TCT GGC CAG TAC ACC CAT GAA
DQA1*00201    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00301    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00401    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*005011   --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*005012   --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*00601    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00701    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00801    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00901    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*01001    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01101    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01201    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*01202    --- -C- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*01301    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---
DQA1*01401    --- --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- ---


                        30                                              40                                              50
DQA1*00101    TTT GAT GGC GAT GAG GAG TTC TAC GTG GAC CTG GAG AAG AAG GAA ACT GTC TGG CGG CTG CCT GTG TTT
DQA1*00201    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00301    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00401    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*005011   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*005012   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00601    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00701    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00801    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00901    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01001    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01101    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01201    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01202    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01301    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01401    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
                                    60                                        70
DQA1*00101   AGC ACA TTT AGA AGT TTT GAC CCA CAG GGT GCA CTG AGA AAC TTG GCT ATA ATA AAA CAA AAC TTG AAC
DQA1*00201   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00301   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --C -G- GC- --- --- --- --- ---
DQA1*00401   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*005011  --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- ---
DQA1*005012  --- --- --- -C- --- --- --- --- --- --- --G --- --- --- --- --- --- -C- --- --- --- --- ---
DQA1*00601   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00701   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- ---
DQA1*00801   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --C -G- GC- --- --- --- --- ---
DQA1*00901   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01001   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- GC- --- --- --- --- ---
DQA1*01101   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- GC- --- --- --- --- ---
DQA1*01201   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- -G- GC- --- --- --- --- ---
DQA1*01202   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- -G- GC--- --- --- --- --- ---
DQA1*01301   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01401   --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---


                                    80
DQA1*00101   ATC ATG ACT AAA AGG TCC AAC CAA ACT GCT GCT ACC AAT
DQA1*00201   --- --- --- --- --- --- --- A-- --- --- --- --- ---
DQA1*00301   --- C-- --- --- --T --- --- --- --- --- --- --- ---
DQA1*00401   --- C-- --- --- --- --- --- --- --- --- --- --- ---
DQA1*005011  --- --- --- --- --- --- --- A-- --- --- --- --- ---
DQA1*005012  --- --- --- --- --- --- --- A-- --- --- --- --- ---
DQA1*00601   --- C-- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00701   --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00801   --- C-- --- --- --- --- --- --- --- --- --- --- ---
DQA1*00901   --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01001   --- C-- --- --- --T --- --- --- --- --- --- --- ---
DQA1*01101   --- --- --- --- --- --- --- --- --- --- --- --- ---
DQA1*01201   --- C-- --- --- --T --- --- --- --- --- --- --- ---
DQA1*01202   --- C-- --- --- --T --- --- --- --- --- --- --- ---
DQA1*01301   --- --- --- --- --- --- --- A-- --- --- --- --- ---
DQA1*01401   --- --- --- --- --- --- --- --- --- --- --- --- ---
```

Fig. 5.2. DLA-DQA1 amino acid alignment. HVR represents the hyper variable regions

previously recognized in Kennedy et al. (2000).

```
                    10          20          30          40          50          60          70          80      87
DQA1*00101   DHVAN YGINVYQSYG PSGQYTHEFD GDEEFYVDLE KKETVWRLPV FSTFRSFDPQ GALRNLAIIK QNLNIMTKRS NQTAATN
DQA1*00201   ----Y ---------- ---------- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*00301   ----Y ---------- ---------- ---------- ---------- ----T----- ------RA- -----L--S- -------
DQA1*00401   ----Y ---------- ---------- ---------- ---------- ----T----- ---------- -----L---- -------
DQA1*005011  ----Y ---------- ----F----- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*005012  ----Y ---------- ----F----- ---------- ---------- ----T----- --------T- ---------- -K-----
DQA1*00601   ----Y ---------- ---------- ---------- ---------- ---------- ---------- -----L---- -------
DQA1*00701   ----Y ---------- ---------- ---------- ---------- ----T----- --------T- ---------- -------
DQA1*00801   ----Y ---------- ---------- ---------- ---------- ----T----- -------RA- -----L---- -------
DQA1*00901   ----Y ---------- ----F----- ---------- ---------- ---------- ---------- ---------- -------
DQA1*01001   ----Y ---------- ---------- ---------- ---------- ---------- -------A- -----L--S- -------
DQA1*01101   ----Y ---------- ---------- ---------- ---------- ----T----- -------A- ---------- -------
DQA1*01201   ----Y ---------- ----F----- ---------- ---------- ----T----- -------RA- -----L--S- -------
DQA1*01202   -P--Y ---------- ----F----- ---------- ---------- ----T----- -------RA- -----L--S- -------
DQA1*01301   ----Y ---------- ----F----- ---------- ---------- ----T----- ---------- ---------- -K-----
DQA1*01401   ----Y ---------- ----F----- ---------- ---------- ----T----- ---------- ---------- -------
                             |                                |           |-- ---------- -|
                           HVR 1                            HVR 2            HVR 3
```

differed at nucleotide position 22 (Fig. 5.1). This polymorphism resulted in a non-synonymous change outside the hypervariable region. Only one clone was sequenced for this allele, therefore further confirmation is needed to verify the presence of this new allele.

Among the 16 DQA1 alleles, eight of the nine variable amino acid positions within the antigen recognition site resulted in a nonsynonymous variation. The rate of non-synonymous (d_N) and synonymous (d_S) substitutions was estimated for both the ABS and non-ABS amino acid positions in Table 5.1. In both the antigen and non antigen binding site positions, d_N is significantly greater than d_S, with an average ratio d_N / d_S of 3.5.

Based on the amino acid sequence, we used the program MEGA (Kumar et al. 1993) to construct a neighbour-joining tree (Fig. 5.3). This tree includes the 11 DQA1 sequences previously published plus the five new alleles identified in this study. Only one branch had a bootstrap value greater than 75%. This lineage includes the two alleles currently confined to the Texas coyote population. A nonsynonymous nucleotide variation outside the hypervariable region is the only difference between these two alleles (Fig. 5.1 and 5.2). The alleles found in all four species are dispersed throughout the tree.
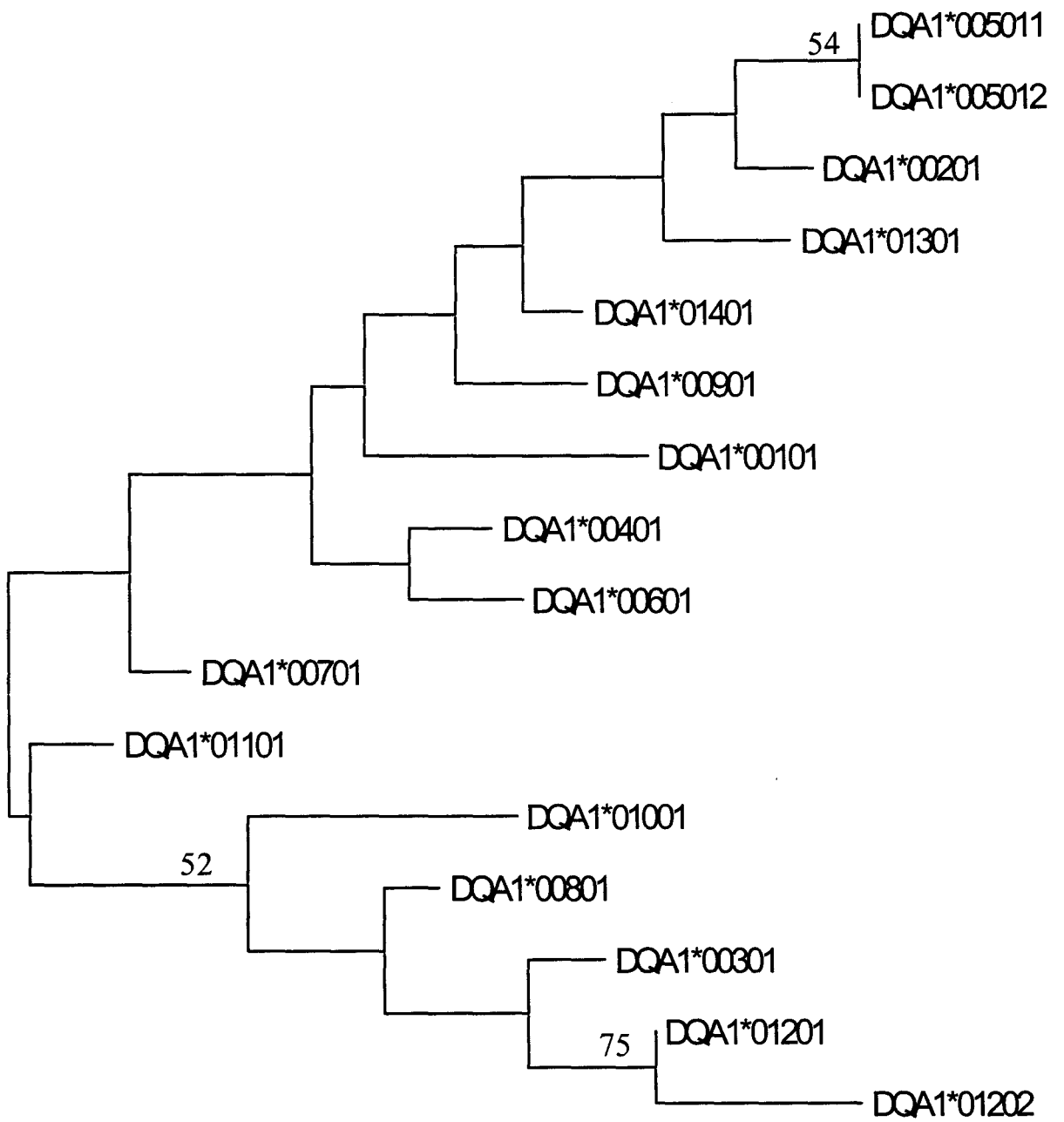
Many of the alleles were shared between the four species, however the most common allele in each of the species was different (Table 5.2). The most common alleles in the gray wolf, the eastern timber wolf, the red wolf and the western coyote were DQA1*00101 (0.225), DQA1*00511 (0.396), DQA1*01101 (0.500) and DQA1*00901 (0.2500) respectively. Among the four species, fifteen of the 71 animals analyzed were homozygous. Five percent of the red wolf animals analyzed were homozygous, which is

Table 5.1. The estimated rates of nonsynonymous and synonymous substitutions for antigen and nonantigen binding amino acid positions and their ratio, where N is the number of codons in each category. Standard errors are in parentheses.

Positions	N	d_N	d_S	d_N / d_S
Antigen Binding	17	0.072 (0.028)	0.024 (0.018)	3.00
Nonantigen binding	65	0.002 (0.001)	0.000 (0.000)	∞
All	82	0.021 (0.008)	0.006 (0.005)	3.500

Fig. 5.3. A neighbour joining tree based on the Jukes and Cantor (1969) distance method giving the relationship for the 16 sequences found among all North American canis species. Boostrap values were calculated using 1000 replicates. Bootstrap values are indicated at nodes if found in more than 50% of 1000 bootstrap trees.

DQA1*005011

54

DQA1*005012

DQA1*00201

DQA1*01301

DQA1*01401

DQA1*00901

DQA1*00101

DQA1*00401

DQA1*00601

DQA1*00701

DQA1*01101

DQA1*01001

52

DQA1*00801

DQA1*00301

75 DQA1*01201

DQA1*01202

0.005

Table5.2. The observed frequencies of the 12 alleles identified in four species of North America, where N is the sample size.

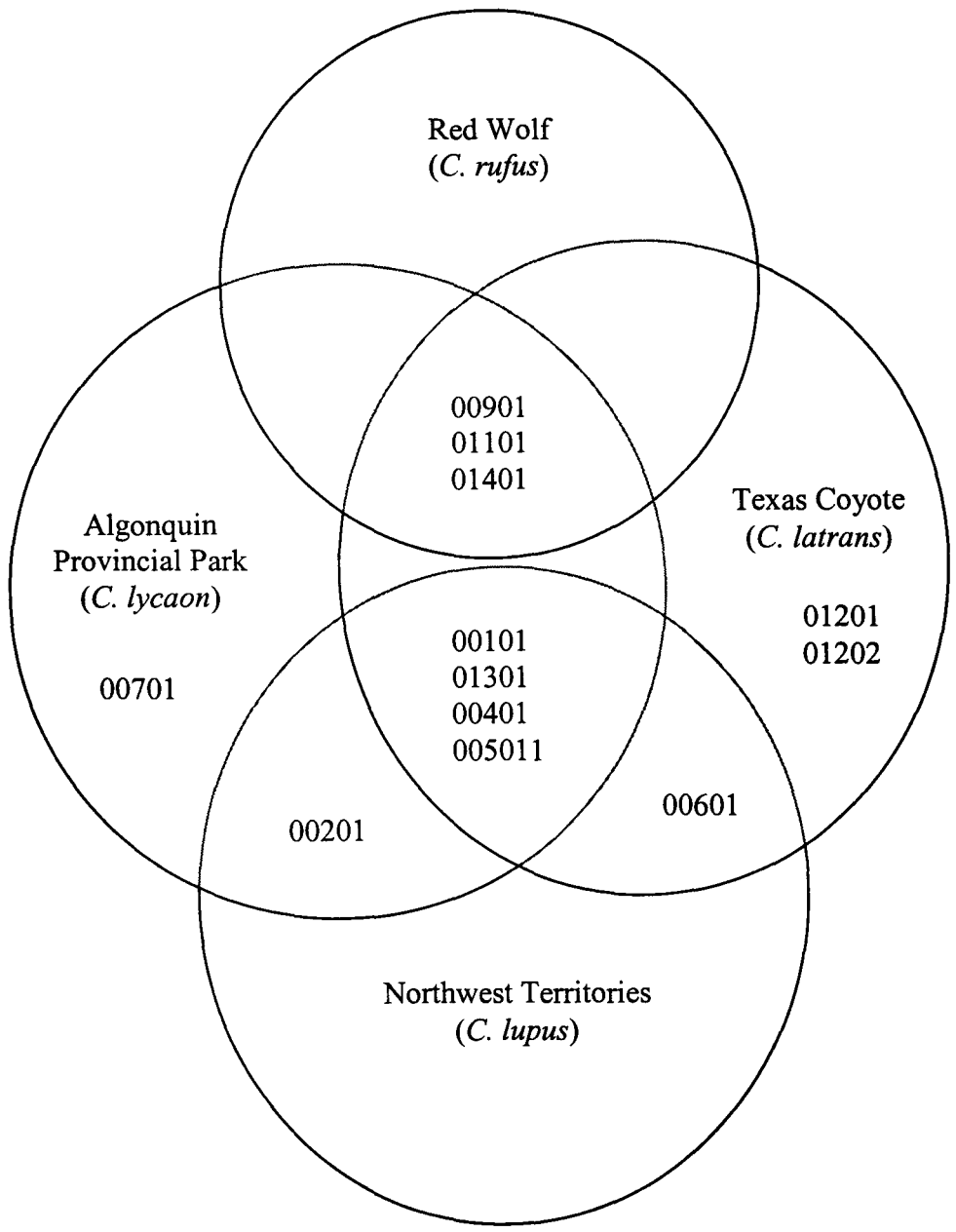| DLA-DQA1 Allelic designations	Northwest Territories *Canis lupus* (N=20)	Algonquin Provincial Park *Canis lycaon* (N=24)	Captive Red Wolf *Canis rufus* (N=7)	Texas Coyote *Canis latrans* (N=20)
DQA1*00101	0.225	0.188	0.000	0.125
DQA1*00201	0.075	0.083	0.000	0.000
DQA1*00401	0.075	0.021	0.000	0.025
DQA1*005011	0.125	0.396	0.000	0.050
DQA1*00601	0.125	0.000	0.000	0.025
DQA1*00701	0.000	0.021	0.000	0.000
DQA1*00901	0.000	0.145	0.286	0.250
DQA1*01101	0.000	0.021	0.500	0.125
DQA1*01201	0.000	0.000	0.000	0.050
DQA1*01202	0.000	0.000	0.000	0.050
DQA1*01301	0.075	0.062	0.000	0.050
DQA1*01401	0.000	0.021	0.214	0.150
Undetermined	0.300	0.042	0.000	0.100

surprising given that the captive breeding program began with 14 founders. Of the heterozygotes, the allelic profiles of nine individuals could not be determined as they contained unsequenced alleles. If each animal had one of the common alleles, a minimum of 4 new alleles could account for all nine heterozygotes.

The alleles shared among the species are shown in Fig. 5.4. Two alleles were found exclusively in the Texas coyote population (DQA1*01201 and DQA1*01202). One allele (DQA1*00701) previously identified in dogs was found in one Algonquin park wolf. No new alleles were exclusive to the red or gray wolf. Three alleles were shared among *C. latrans*, *C. rufus* and *C. lycaon*, of which two were not previously identified in domestic dogs. Four alleles were shared among *C. latrans*, *C. lycaon* and *C. lupus*, of which one was not previously identified in dogs. One allele was shared between *C. latrans* and *C. lupus* and another was shared between *C. lycaon* and *C. lupus*. Both alleles were identified previously in domestic dogs.

Among the geographic populations there are some allelic differences. The most common allele, DQA1*00511 found in Algonquin was low to absent in the Texas coyote and red wolf populations. The frequency of that allele in Algonquin was more similar to that of the gray wolf population and could therefore be more characteristic of a larger wolf. In contrast the more common allele, DQA1*00901 identified in the Texas coyote population was found in the captive red wolf and the Algonquin populations. This allele was absent in the larger northern gray wolf samples. Similarly allele DQA1*01401 was found at a similar frequency in the Texas coyote and red wolves samples. However it was

Fig. 5.4. Venn diagram describing the shared and unshared status of DLA-DQA exon 2 alleles among four populations of wolves and coyotes in North America. Alleles are designated according to the dog nomenclature (Kennedy et al. 2000) and listed in table 1. For visual purpose the DQA1* prefix of each of the alleles is left out in this figure. Each circle represents the population indicated by the labels. Shared sequences are found in the intersection of circles whereas sequences present only in one population are found only in one circle (see results section).

Red Wolf
(*C. rufus*)

Algonquin
Provincial Park
(*C. lycaon*)

Texas Coyote
(*C. latrans*)

Northwest Territories
(*C. lupus*)

00901
01101
01401

00701

01201
01202

00101
01301
00401
005011

00201

00601

present at a low frequency in the Algonquin population and absent from the northern gray wolf population.

DISCUSSION

Five new DLA-DQA1 alleles were identified in this study. Two were found only Texas coyotes, another two were found only in the eastern timber wolf, the red wolf and the western coyote and a fifth was shared by the eastern timber wolf, the western coyote and the gray wolf. In addition to the five new DQA1 alleles identified in this study, seven previously published alleles found in dog breeds (Sarmiento et al. 1992, Wagner et al. 1995; Polvi et al. 1997, Kennedy et al. 2000) were identified among the four populations. All alleles were shared between at least three of the species, except for DQA1*01201 and DQA1*01202 which were found only in the Texas coyote population and DQA1*00201 and DQA1*00601 (found also in dogs), which were shared between *C. lycaon* and *C. lupus* and *C. latrans* and *C. lupus* respectively.

The 12 alleles found in the four *Canis* species were dispersed throughout the phylogenetic tree. The absence of species-specific grouping of alleles within the tree is not uncommon for MHC genes. Based on the trans species transmission of MHC alleles (Klein 1987; Klein and Takahata 1990), variants now found in a species originally came from a group of alleles that was passed down from an ancestral species. Therefore, alleles from different species are often more related to one another than to alleles from the same species. Some studies (McConnell et al. 1988; Lawler et al. 1988) have shown common alleles found in species that have diverged 1-10 million years ago. In this study the two

amino acid sequences (DQA1*01201 and DQA1*01202) confined to the Texas coyote population are more similar to alleles found in various dog breeds than with other alleles found in western coyotes. The common ancestor between coyotes and the domestic dog is estimated to date back more than a million years (Wayne et al. 1991).

It has been suggested that the high level of MHC polymorphism is linked to pathogen-based selection (Klein 1987, Potts and Slev 1995), primarily due to the involvement of MHC genes in the immune response. By possessing a range of MHC alleles, an individuals' ability to initiate an immune response is heightened because of the larger array of foreign antigens it can bind. An individual that lacks a specific MHC allele may be unable to initiate an immune response to the invading parasite or pathogen. This suggests some sort of balancing selection is involved, perhaps through heterozygote advantage or overdominance (Nei 1987) with some influence of frequency-dependent selection.

It has been suggested (Nowak 1979, Wayne et al. 1991, Wilson et al. 2000), that the common ancestor of the Eurasian-evolved gray wolves and North American-evolved wolves and coyotes lived approximately 1-2 million years ago. All of the domestic dogs appear to have originated from the Eurasian gray wolf. Recent genetic data (Wilson et al. 2000) has been interpreted to suggest that the eastern timber wolf is closely related to the red wolf and the divergence of these and the western coyote is estimated at approximately 150,000-300,000 years ago. It is therefore not surprising that these species share many alleles. Hybridization between the species would have resulted in even more sharing of alleles. The red wolf and the eastern timber wolf readily hybridise with the western

coyote and less readily with the gray wolf. There is no strong evidence that the western coyote hybridizes with the gray wolf, even the small Mexican wolf.

Although there are not species-specific alleles, certain alleles are more common in the different species. The most common allele in each is different and the allele frequencies differ considerably. Further screening of individuals in the four species and other populations is needed.

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Chapter 6

GENERAL DISCUSSION

This thesis set out to achieve two major goals: 1) to identify the evolutionary relationship of wolves and coyotes in North America using functional and non functional genetic markers and 2) to characterize and assess the relationship of animals within Algonquin Provincial Park to those in the surrounding populations of wolves and coyotes.

The controversy over the taxonomy of wolves in North America has been a long-standing one. It is agreed in the literature that an ancestral wolf-like canid inhabited North America approximately 2 million years ago (Nowak 1979; Nowak 1995). Individuals of this group emigrated to Eurasia at this time, and evolved into the gray wolf, *C. lupus*, which then migrated back to North America via the Bering land bridge during the Pleistocene ice age, approximately 300,000 years ago (Nowak 1979; Kurten & Anderson 1980). Over the last century the classification of wolves in North America has been controversial ranging from a distinction of 24 subspecies to the present classification of five (Nowak 1995). Included among the five gray wolf subspecies is the eastern timber wolf. Not included is the red wolf, *C. rufus*, which maintains its species designation despite ongoing debates.
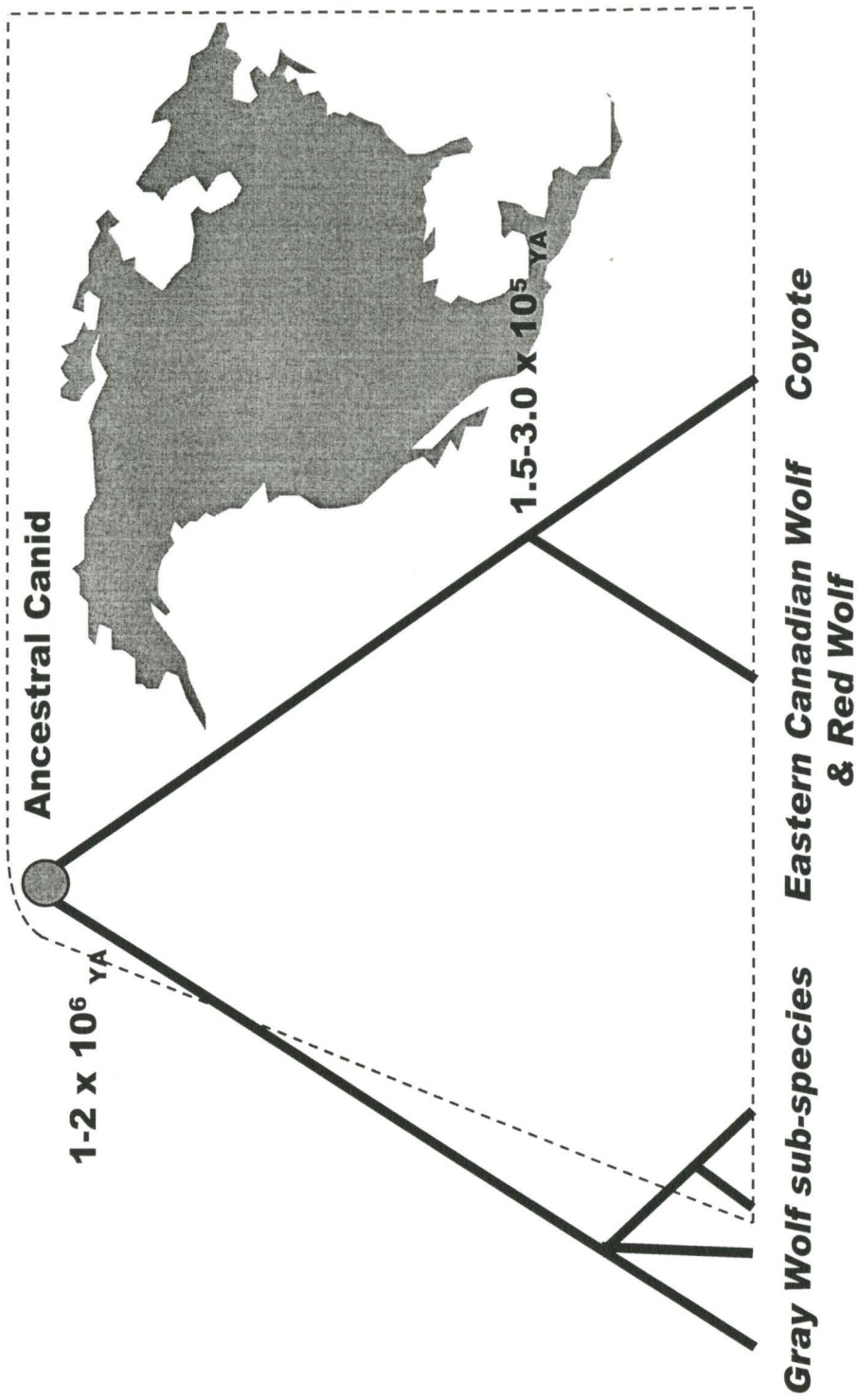
It was not until our initial genetic study on Algonquin Park wolves, that a similarity between the eastern wolf and the red wolf was identified. The genetic data

presented in this thesis supports a new evolutionary model (Figure 6.1), which suggests

that the eastern timber wolf and the red wolf diverged in North America from the coyote

within the mid-Pleistocene, 150,000-300,000 years ago. Furthermore, the evolution of

North American wolves and coyotes occurred independently of the gray wolf, *C. lupus*,

which evolved in Eurasia 1-2 million years ago. The data presented leads to the formal

rejection of the hypothesis that the red wolf and the eastern timber wolf are hybrids of

coyotes and gray wolves. In addition, the data leads to the rejection of the hypothesis that

the eastern timber wolf is a subspecies of the gray wolf. At present the red wolf is

classified as a separate species, *C. rufus*, however, based on historical taxonomic

classifications, the eastern North American wolves would require the original designation

of *C. lycaon*.

Assuming the proposed taxonomic revision is accepted, the findings in this thesis

have broader biological, ecological and conservation implications. Currently the largest

protected population of the eastern timber wolf is in Algonquin Provincial Park, Ontario,

Canada. Using genetic data and a number of clustering analyses the present range of the

North American-evolved eastern timber wolf was assessed. We compared our findings to

a previous identification of four wolves in Ontario by Kolenosky and Standfield (1975);

(1) *C. lupus hudsonicus*, a subspecies of the gray wolf inhabiting the sub-arctic tundra.

(2) An "Ontario type" of a second subspecies of the gray wolf, the eastern timber wolf (*C. l. lycaon*) that inhabits the boreal forests and much of the Hudson Bay Lowlands. (3) A

second "race" ("Algonquin type") of *C. l. lycaon* that inhabit the deciduous forests of the

upper Great Lakes. And (4) A small wolf ("Tweed type") that has been proposed to be a

Figure 6.1. A model for the evolution of North American wolves. The progenitor to *C. lupus, C. lycaon* and *C. latrans* is indicated at the top. Divergence from this ancestor is generally accepted to have occurred 1-2 million years ago when the progenitor of *C. lupus* migrated to Eurasia. The North American species diverged 150,000-300,000 years ago into the eastern Canadian wolf /red wolf (*C. lycaon*) and the coyote (*C. latrans*). Recently, *C. lycaon* and *C. latrans* have come into contact and have subsequently hybridized. The Eurasian-evolved *C. lupus* returned to North America within the Pleistocene.

Ancestral Canid

1-2 x 10⁶ YA

1.5-3.0 x 10⁵ YA

Gray Wolf sub-species

Eastern Canadian Wolf
& Red Wolf

Coyote

hybrid between the "Algonquin type" wolf and the expanding population of western coyotes, *C. latrans*. We also examined the amount of inter-breeding between *C. lupus* and *C. lycaon* and *C. lycaon* and *C. latrans* in Ontario to assess the genetic composition and distribution of these animals across the province.

The genetic data support the hypothesis that the "Tweed wolf" is a hybrid between the coyote and eastern timber wolf (*C. latrans* and *C. lycaon*). Despite the high numbers of the "Tweed" animals, southeast of Algonquin Park, the data suggests barriers to gene flow exist thus maintaining larger wolf-like animals within the Park. The eastern timber wolf in Algonquin Park represents the "Algonquin" type described by Kolenosky and Standfield (1975). Despite limited gene flow from the Frontenac Axis, a high level of genetic variation is present in Algonquin Park. This variability is supported by the gene flow from the Magnetawan region, northern Ontario and Quebec. There is evidence to suggest a range in size of animals across Ontario, which appears to be primarily due to the introgression of more coyote genetic material in the south and more gray wolf genetic material in northern Ontario. The "Ontario type" animal as described by Kolenosky and Standfield (1975) appears to be in Pukaskwa National Park. This small population of gray wolves, (*C. lupus*), which prey on moose, appear to be surrounded by the larger "Algonquin type" animals in patchy habitat that contains moose and white-tailed deer. The broad band across northeastern and central Ontario, which Kolenosky and Standfield (1975) described as the area where the "Ontario" and "Algonquin" types meet, appears to contain *C. lycaon* animals impacted by *C. lupus* genetic material.

The genetic connectivity between Algonquin Park animals and those to the north and

west supports the conclusion that the population of *C. lycaon* in Ontario is large, numbering in the thousands rather than the hundreds. The connectivity between geographic groups of the eastern timber wolf and the high variation in the Park animals suggests Algonquin is not an island population, but the southern part of a larger metapopulation of *C. lycaon*, that stretches from Quebec to Manitoba and which may include the animals in Minnesota, Wyoming and Michigan.

Algonquin Provincial Park

Algonquin Provincial Park maintains the largest protected population of the eastern timber wolf. The information that can be obtained from the wolves in the Park is important to our understanding of the eastern wolf and its social structure. Few studies have had the ability or opportunity to collect samples and record observations over a long-term study. The data collected over the 12 years of study on wolf packs in Algonquin Park is unique and has enhanced our knowledge about the pack social structure of the eastern timber wolf. The data suggests that wolf packs are not composed of simply an unrelated breeding pair and their offspring, but that in the majority of packs numerous subordinates are present. The data further suggests that subordinates merge into packs through a complex number of mechanisms including, pack adoption, pack splitting, dispersal and immigration. Although the data are limited, it appears that pack territory may be passed on from father to son. Females appear to be the major dispersers. Whether these results are typical of the eastern timber wolf or due to the high mortality observed in the Park, we are unable to determine.

The high mortality observed in the Park due to the seasonal migration of wolves outside the boundaries of Algonquin has been raised as a concern for the long-term persistence of wolves in Algonquin Park. In addition the possible introgression of western coyote genetic material into Park animals has also been viewed as a threat. Although our data are consistent with the idea that the mortality may be responsible for the observed complexity of wolf social structure, the data do not support the suggestion that coyote introgression is a present threat.

Algonquin Wolf Advisory Group

The apparent decline in wolf numbers in Algonquin Provincial Park, was responsible for the initial formation of the Algonquin Advisory Group (AWAG) by the Minister of Natural Resources, John Snobelen in 1998. The purpose of this group was to investigate the status of the wolves in the Park and recommend management actions for long-term persistence. Twenty-four recommendations for a conservation plan on wolves in Algonquin Provincial Park were made. Many of the recommendations involved increasing public awareness of wolves and limiting hunting around the Park, for which a 30-month moratorium in 39 townships has been proposed. Other recommendations involved reducing forest crown cover and providing no interference with natural disturbances, such as wildfire, unless it directly affects human safety.

Concluding Remarks

Algonquin Provincial Park has gone through a number of changes since its

formation. The original ecosystem, was changed in the 1800s as a result of deforestation and wildlife control. With new policies and concern for our environment, management strategies have led to a reversion of the Park ecosystem closer to its original state. Conserving the biodiversity of Algonquin Provincial Park is an important part of the Park's mandate. Two strategies for maintaining a top end predator in Algonquin are possible: 1) Allow the Park to evolve with minimal management, thereby occupying a larger eastern timber wolf as a top end predator 2) Manage the Park for deer and the present small eastern timber wolf. Based on the recommendations by AWAG, it appears that the committee did not adopt either strategy. The main issue that their recommendations have dealt with is wolf mortality. Therefore this suggests that the group has not met its original task "...to ensure the long term conservation of these wolves" (AWAG 2001). Based on the data presented in this thesis and with evidence of the introgression of *C. lupus* from the north to the south the recommended strategy should be to minimize management, which would allow for the natural evolution of a larger eastern timber wolf. Evidence of geneflow between the Park and areas north of Lake Superior suggest corridors can be maintained for the long-term conservation of the larger eastern timber wolf as a top end predator in Algonquin Provincial Park.

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