GENETIC CHARACTERIZATION OF *CANIS* POPULATIONS IN THE WESTERN GREAT LAKES REGION

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ABSTRACT

Genetic characterization of *Canis* populations in the western Great Lakes region

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The genetic status of *Canis* populations in the western Great Lakes Region (WGLR) has been debated for years. This thesis examines the genetic composition of *Canis* in the WGLR to determine which *Canis* species contributed to the genomes of extant and pre-recovery WGLR wolf populations. Analysis of microsatellite genotypes, and mitochondrial DNA and Y-chromosome sequences, revealed that the majority of the extant WGLR canids sampled are part of a large wolf population extending across Michigan, Wisconsin, Minnesota and northwestern Ontario, having predominantly eastern wolf (*C. lycaon*) and gray wolf (*C. lupus*) ancestry. The extant WGLR wolf population was determined to be composed of gray-eastern wolf hybrids that do not breed with sympatric coyotes (*C. latrans*). Genetic profiles of three century-old wolves from the WGLR were similar to those of extant animals in the region, and this was interpreted to suggest that current WGLR wolves are representative of the pre-recovery population.

Keywords: hybridization, eastern wolf, gray wolf, coyote, mitochondrial haplotype, microsatellite genotype

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Chapter 1: General Introduction

Overview

Hybridization between species of the genus *Canis* has been clearly documented based on morphological and genetic data (e.g. Wayne and Vila 2003). The most notable example of *Canis* species hybridization occurs between wolves and coyotes in northeastern North America (e.g. Lehman *et al.* 1991; Wilson *et al.* 2008). Hybridization between *Canis* species has led to issues regarding the taxonomic distinction of wolf and coyote populations and has affected their management and conservation because of: 1) inaccurate assessment of relative population sizes; 2) difficulty enforcing the protection of species that have hybridized with non-protected species, given the lack of definitive hybrid policies; and 3) uncertainty regarding whether hybrids warrant protection (e.g. natural versus human-caused hybridization). Presently, hybridization may be the most important issue regarding the conservation and management of wolves in North America (e.g. Wayne and Vila 2003; Leonard and Wayne 2008).

Hybridization

Hybridization is generally defined as the "interbreeding of hetero-specific individuals from genetically distinct populations regardless of the taxonomic status of the populations", but hybridization has also been used to describe matings between individuals of different subspecies or populations, which although they may be taxonomically indistinguishable, can differ genetically (Rhymer and Simberloff 1996). When hybridization among species/populations is extensive and ongoing, hybrid swarms may develop, which are populations of individuals in which "introgression has occurred

to various degrees by varying numbers of generations of backcrossing to one or both parental taxa, in addition to mating among the hybrids themselves" (Rhymer and Simberloff 1996). Hybridization is an important issue for researchers because it can have significant implications for the conservation and management of threatened or endangered species.

Hybridization can be a natural evolutionary process, but it can also be exacerbated by human activities such as habitat modification, fragmentation, and the introduction of non-native plants and animals (Allendorf *et al.* 2001). The interaction of these activities results in the breakdown of geographic barriers and reproductive isolation between allopatric species, allowing them to interbreed (Fox 2008). The processes of habitat modification and fragmentation homogenize natural environments, removing ecological niches and promoting hybridization (Fox 2008). Species may be more susceptible to hybridization when operational sex ratios are strongly skewed or in areas where population density of conspecifics is low, i.e. near their distribution fringe (Reyer 2008). Hybrid zones may develop at the contact point of two species and progressively expand into their respective distributions given continued hybridization.

Hybridization is generally regarded as a process that reduces biodiversity, however, it can also create biodiversity by combining traits of species and facilitating evolutionary adaptation. Hybridization can restore genetic variation to populations that have lost variation due to bottlenecks, genetic drift or inbreeding, however, hybridization can also be a threat to genetically distinct populations (e.g. gene swamping of red wolves by coyotes: Wayne and Vila 2003; Waits and Murray 2007; Kyle *et al.* 2008).

Hybridization has generally been assessed based on the presence of phenotypic features, morphology or behavior intermediate to the hybridizing taxa, however, hybrid individuals are not always intermediate to the parents based on these measures of differentiation (Allendorf *et al.* 2001). Advances in molecular genetics have allowed researchers to identify and quantify levels of genetic admixture between hybridizing species/populations for a variety of taxa: wildcats and domestic cats (Pierpaoli *et al.* 2003); mottled ducks and mallards (Williams *et al*. 2005); lynx and bobcats (Schwartz *et al.* 2004); chukars and partridges (Barilani *et al.* 2007); rainbow trout and cutthroat trout (Young *et al.* 2001); wolves and dogs (Andersone *et al.* 2002; Randi and Lucchini 2002); eastern wolves and coyotes (Lehman *et al.* 1991, Roy *et al.* 1994, Wilson *et al.* 2008).

In North America, where ranges of several *Canis* species overlap, various degrees of hybridization over time has led to a continuum of genetic and morphological differences throughout the landscape, perhaps most notably in northeastern North America (e.g. Wayne and Vila 2003). The Federal Wildlife Service in the United States has no official position on wolf-coyote hybrids but actions suggest protection is not considered warranted (Adkins Giese 2006). The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) designated the eastern wolf (*Canis lycaon*) a species of special concern federally in 2001 under the Species at Risk Act, recognizing that the eastern wolf may be threatened by hybridization with coyotes (COSEWIC 2006). Similarly, the Committee on the Status of Species at Risk in Ontario (COSSARO) designated the eastern wolf a species of special concern in 2004 on the Species at Risk in Ontario List, recognizing that eastern wolves have hybridized with both gray wolves and coyotes (OMNR 2005). Presently, wolf-coyote hybrids have no official federal status to

warrant protection in Canada (COSEWIC 2006), but they are protected in Ontario under the *Fish and Wildlife Conservation Act* (1997) (OMNR 2005).

Canis Taxonomy and Genetics

There has been much debate concerning the taxonomic status of *Canis* species in North America over the past century. Previous taxonomic designations were largely based on morphological differences and variation in phenotypic traits (Miller 1912; Pocock 1935; Goldman 1944; Nowak 1979), notably resulting in disagreement over the number of gray wolf (*Canis lupus*) subspecies (Hall 1981; Nowak 1995). Genetic studies conducted over the last two decades have provided further insight into the evolutionary history of *Canis* species, both regionally and globally (see Wayne and Vila 2003). It is generally accepted that the progenitor of the gray wolf migrated from North America to Eurasia and evolved there approximately 1 to 2 million years ago, and later returned to North America via the Bering land bridge (Nowak 1979; Lehman *et al.* 1991; Wilson *et al.* 2000). Based on genetic evidence the eastern wolf (*Canis lycaon*) was shown to be a distinct species, conspecific to the red wolf (*Canis rufus*), that evolved in North America sharing a common lineage with the western coyote (*Canis latrans*) until 150 000 to 300 000 years ago (Wilson *et al.* 2000) (Figure 1.1). Early taxonomic descriptions had characterized the eastern wolf as a distinct species *C. lycaon* (Miller 1912; Pocock 1935), however it was later designated a subspecies of the gray wolf, *C. lupus lycaon* (Young and Goldman 1944), and gray wolf subspecies status was maintained until recently (Wilson *et al.* 2000; Kyle *et al.* 2006).

FIGURE 1.1.

Prior to European settlement of North America, the gray wolf was thought to occupy most of the continent with the exception of the eastern deciduous forests (Nowak 1995), where a morphologically distinct species of wolf was recognized (Young and Goldman 1944; Peterson 1966; Kolenosky and Standfield 1975; Theberge 1991; Nowak 2002), representing the eastern wolf. Coyotes were historically restricted to the plains and deserts of central North America (Bekoff and Wells 1986; Moore and Parker 1992). Widespread deforestation and conversion of land to agriculture occurred in North America following European settlement, and as agriculture advanced to the northwest the number of wolves declined severely due to habitat destruction, fur harvests, reductions in the numbers of ungulate prey, and extermination by humans through predator control programs (Young and Goldman 1944; Nowak 2002). The gray wolf was extirpated from southeastern Ontario between 1850 and 1900 (Kolenosky and Standfield 1975), and soon after eastern wolves extended their range northward, apparently following the northward range expansion of white-tailed deer (Kolenosky and Standfield 1975; Nowak 2002 & 2003). Declines in the number of wolves coupled with the ability of coyotes to persist in agricultural areas and colonize disturbed habitat allowed coyotes to expand their range into southeastern Canada in the early 1900s (Nowak 1979).

Habitat and landscape conditions have been implicated in the reduction of the range of wolves and the expansion of the coyote's range northward in eastern North America (Nowak 1978; Moore and Parker 1992), as well as extensive hybridization between eastern wolves and coyotes in northeastern North America (Lehman *et al.* 1991; Wayne *et al.* 1992; Roy *et al.* 1994; Wilson *et al.* 2008). The movement of coyotes into northeastern North America in the early 1900s was followed by hybridization between

coyotes and eastern wolves (Silver and Silver 1969; Kolenosky and Standfield 1975). Extensive hybridization between *C. lycaon* and *C. latrans* in northeastern North America has led to a hybrid species referred to as the "Tweed wolf" or eastern coyote (*Canis latrans X lycaon*) (Kolenosky and Standfield 1975; Sears *et al.* 2003; Wilson *et al.* 2008), which includes individuals that are morphologically and phenotypically intermediate between *C. lycaon* and *C. latrans*, and contain differing proportions of those species' genetic material. Hybridization between eastern wolves and coyotes appeared to involve asymmetric breeding (Lehman *et al.* 1991), whereby male wolves bred with female coyotes (Pilgrim *et al.* 1998). However, extensive and ongoing hybridization has obscured sex-biased mating between wolves and coyotes because of backcrossing of hybrid progeny, which has led to putative hybrid swarms (e.g. Wilson *et al.* 2008). No direct hybridization has been observed between gray wolves and coyotes in western populations (Pilgrim *et al.* 1998; Hailer and Leonard 2008), nor has direct hybridization been observed between Mexican gray wolves and coyotes (Garcia-Moreno *et al.* 1996; Hedrick *et al.* 1997; Hailer and Leonard 2008). The ability of eastern wolves to hybridize with coyotes is likely the result of their close evolutionary relationship (Wilson *et al.* 2000) whereas the more distantly related gray wolves do not breed with coyotes, however, there is evidence that eastern wolves and gray wolves hybridize (Mech and Federoff 2002; Wilson *et al.* 2008).

It is suggested that prior to European settlement the eastern wolf was separated from gray wolves and coyotes by glacial barriers (Nowak 2002) that were subsequently maintained by reproductive barriers due to habitat and prey specificities of the three species (Kolenosky and Standfield 1975; Moore and Parker 1992; Nowak 1995; Geffen

et al. 2004). Agricultural and forestry practices along with predator control programs are suggested to have resulted in the breakdown of reproductive barriers between gray wolves and eastern wolves and between coyotes and eastern wolves in the northwestern and southeastern portions of the eastern wolf's range, respectively (Kyle *et al.* 2006). Putatively, this has led to what we know today as the "eastern wolf" being composed of individuals exhibiting a gradient of morphology and genetic composition across its range, with more coyote genetic material being observed in the southeastern portion of its range, and more gray wolf genetic material observed in the northwestern portion of its range (Kyle *et al.* 2006). The eastern wolf may be viewed as the mediator of hybridization among *Canis* species in North America, and this is supported by the lack of hybridization in western *Canis* populations where *C. lycaon* is absent (e.g. Pilgrim *et al.* 1998).

Wolves are highly mobile and capable of dispersing long distances (Fritts 1983; Mech 1987), and their ability to traverse many landscapes and potential topographic barriers (e.g. rivers, mountains) minimizes the influence of geographical factors on gene flow and masks the effects of historical events, making the effects of contemporary ecological factors on genetic differentiation more prominent (Pilot *et al.* 2006). However, when studying the distribution of *Canis* species, it is important to consider the historical broad scale patterns of colonization that have contributed to the present distributions of pure and hybrid *Canis* species. The present distribution of *Canis* reflects a combination of contemporary selective forces and historic colonization patterns, as well as recent colonization events and human factors such as landscape changes and wolf control programs. Ecological barriers to dispersal can also be important in shaping the distribution of *Canis* (e.g. Carmichael *et al.* 2001; Musiani *et al.* 2007).

Molecular Genetic Techniques for Studying Canis

The majority of early genetic studies of *Canis* were based on analyses of the mitochondrial DNA (mtDNA) control region, which is inherited maternally and clonally (i.e. lack of recombination) in mammals, and therefore can be used to detect past hybridization events (Pilgrim *et al.* 1998). Genotyping of microsatellite loci has led to a better understanding of the evolutionary relationships and hybridization patterns of *Canis* species, and has allowed for greater genetic differentiation at the level of the population and the individual (Roy *et al.* 1994; Wilson *et al.* 2000). Because of the high degree of polymorphism and evolutionary rate of microsatellite loci, they are very informative for describing gene flow and hybridization (Roy *et al.* 1994, Wilson *et al.* 2008). Analysis of the Y-chromosome in male *Canis* individuals can provide information on paternal gene flow, complementing mtDNA data to detect asymmetric breeding between species and determine the role of each sex in natural processes (Sundqvist *et al.* 2001). The combined information from these three types of genetic markers can achieve increased resolution in differentiating among populations or individuals, and facilitates a greater ability to describe and quantify the degree of admixture between species, as well as the directionality and temporal context of hybridization.

Objective of Thesis

Amidst the controversy over North American *Canis* taxonomy, the situation in the western Great Lakes region (WGLR) warrants special attention. Due to killing by humans and reductions in prey abundance, wolf populations were extirpated from Wisconsin and Michigan by 1960, and were eliminated from most of Minnesota,

persisting only in the remote northeastern portion of the state (FWS 2007). At the same time wolves persisted across most of northern Ontario owing to the relatively vast expanses of uninhabited lands in this province. Since gaining protection under the Endangered Species Act in 1974, wolf populations in the western Great Lakes states have recovered to substantial enough numbers to facilitate the recent decision to de-list wolves as endangered/threatened in the Western Great Lakes Distinct Population Segment (FWS 2007). However, the taxonomic status and genetic identity of the current WGLR wolf population is uncertain, with some studies suggesting the animals are hybrids of gray wolves and coyotes (e.g. Lehman *et al.* 1991; Roy *et al.* 1994), and others suggesting that hybrids of eastern wolves and gray wolves may occur in the region (e.g. Mech and Federoff 2002; Wilson *et al.* 2008). The question of whether the recovered WGLR wolf population is representative of the historic (i.e. pre-recovery) population has been raised (Leonard and Wayne 2008), thereby casting doubt on the decision to de-list. With the genetic status of WGLR wolves uncertain in the face of changing conservation and management plans, a focused and detailed genetic study of these animals is necessary.

The overall objective of this thesis is to comprehensively determine the genetic composition of wolves from the WGLR using data from nuclear microsatellite loci, and mtDNA and Y-chromosome sequences. This study aims to clarify the debate over the identity of these canids and to provide further insight into the complex dynamics of *Canis* populations in northeastern North America. The findings of this research will likely have important implications for the conservation and management of *Canis* populations in Ontario and the western Great Lakes states, and broader implications relating to wolf taxonomy in North America.

The major goals of this thesis are: 1) to genetically characterize the animals that comprise the extant WGLR *Canis* population(s) and assess their relationship to populations from surrounding regions; 2) to genetically characterize wolves from the pre-recovery (i.e. historic) WGLR population and compare them to the extant population to determine if the historic population has been restored; and 3) to further assess the range of eastern wolf genetic material in North America.

Based on current knowledge gained from a combination of morphological and genetic studies of *Canis*, it was predicted that 1) wolf populations in the WGLR have not, and do not currently, hybridize with coyotes and 2) the eastern wolf has hybridized with both gray wolves and coyotes, and exhibits a genetic gradient across the landscape. The two central hypotheses of this thesis are that 1) gray wolves and coyotes do not hybridize in wild populations and 2) the eastern wolf is the mediator of *Canis* hybridization in northeastern North America.

This thesis is organized as follows: Chapter two assesses the genetic composition of the extant WGLR population(s) in relation to animals from surrounding regions; Chapter three assesses the genetic composition of historic animals from the WGLR and compares them to extant animals; and Chapter four presents a general discussion to summarize the findings of this thesis and provide a cohesive interpretation of the relations among *Canis* populations in northeastern North America. Recommendations for the conservation and management of *Canis* in northeastern North America are provided based on the findings of this thesis.

Chapter 2: Genetic characterization of contemporary *Canis* populations in the western Great Lakes region using multiple genetic markers

Abstract

The genetic status of *Canis* populations in northeastern North America has been debated for years, and has recently become more important to the conservation of wolves given their recent de-listing in the United States. The genetic composition of *Canis* in the western Great Lakes region (WGLR) and surrounding areas in northeastern North America was examined to determine which *Canis* species contributed to the genomes of extant wolves in the WGLR. Individual genotypes obtained from 12 microsatellite loci were analyzed, and revealed that the majority of the WGLR canids sampled are part of a large homogenous wolf population extending across Michigan, Wisconsin, Minnesota and northwestern Ontario. Mitochondrial DNA and Y-intron sequences were analyzed to determine the maternal and paternal genetic contributions to the current WGLR wolf population, and revealed predominantly eastern wolf and gray wolf ancestry in WGLR canids, with animals having coyote ancestry being distinct from wolves. Thus, based on the three genetic markers used in this study, the WGLR wolf population was determined to be composed of gray-eastern wolf hybrids that do not breed with sympatric coyotes.

Introduction

In northeastern North America, extensive hybridization between *Canis* species has led to a gradient of genetic and morphological differences throughout the landscape (e.g. Kolenosky and Standfield 1975; Wayne and Vila 2003). There has been longstanding debate concerning the taxonomic status of canids in this region, with previous designations being largely based on morphological differences and variation in

phenotypic traits (Miller 1912; Pocock 1935; Goldman 1944; Nowak 1979). It is generally accepted that the progenitor of the gray wolf (*Canis lupus*) migrated from North America to Eurasia and evolved there approximately 1 to 2 million years ago, and later returned via the Bering land bridge (Nowak 1979; Lehman *et al.* 1991; Wilson *et al.* 2000). Based on genetic evidence the eastern wolf (*C. lycaon*) was shown to be a distinct species, conspecific to the red wolf (*C. rufus*), that evolved in North America sharing a common lineage with the western coyote (*C. latrans*) until 150 000 to 300 000 years ago (Wilson *et al.* 2000). The different evolutionary history of these *Canis* species is reflected in their mitochondrial genomes: gray wolves have Old World evolved sequences, and eastern/red wolves and coyotes have New World evolved sequences.

Movement of coyotes into eastern North America in the early 1900s was followed by hybridization between coyotes and eastern wolves (Silver and Silver 1969; Kolenosky and Standfield 1975), which has been extensive and led to the eastern coyote (*Canis latrans X lycaon*) (e.g. Wilson *et al.* 2008). In contrast, no direct hybridization has been observed between gray wolves and coyotes in western populations (e.g. Pilgrim *et al.* 1998; Hailer and Leonard 2008). The ability of eastern wolves to hybridize with coyotes is likely the result of their close evolutionary relationship (Wilson *et al.* 2000), yet there is also evidence that eastern wolves and gray wolves hybridize (Mech and Federoff 2002; Wilson *et al.* 2008). Agricultural and forestry practices along with predator control programs seem to have broken down reproductive barriers between *Canis* species and led to hybridization between coyotes and eastern wolves, and between gray wolves and eastern wolves (Kyle *et al.* 2006). Accordingly, the eastern wolf currently exhibits a gradient of morphology and genetic composition across its range, with more coyote

genetic material observed in the southeastern portion of its range, and more gray wolf genetic material observed in the northwestern portion of its range (Kyle *et al.* 2006). The eastern wolf may be viewed as the mediator of hybridization among *Canis* species in North America, and this is supported by the lack of hybridization in western *Canis* populations where *C. lycaon* is absent (Pilgrim *et al.* 1998). Yet, despite elucidating these patterns of hybridization, considerable uncertainty exists regarding the genetic identity of canids in northeastern North America and the relationships among populations, stressing the importance of genetic research on canids in this region.

The canids of the western Great Lakes region warrant special attention given that by 1960 wolf populations were extirpated from Wisconsin and Michigan, and were eliminated from most of Minnesota (FWS 2007). Wolves have since recovered in the region and were recently removed from protection under the Endangered Species Act in the U.S. (FWS 2007). However, the taxonomic status and genetic identity of the current wolf population is uncertain, with studies suggesting that these canids are either hybrids of gray wolves and coyotes (e.g. Lehman *et al.* 1991; Roy *et al.* 1994; Leonard and Wayne 2008) or hybrids of eastern wolves and gray wolves (e.g. Mech and Federoff 2002; Wilson *et al.* 2008). Given the uncertain taxonomic status of wolves in the western Great Lakes region, a focused and detailed genetic study of these animals was necessary to facilitate effective conservation and management.

The goal of this study was to characterize the genetic attributes of *Canis* in the western Great Lakes region. This investigation is critical because it will elucidate the genetic makeup and attendant conservation status of canids in this region. This understanding is particularly relevant given the recent de-listing of wolves in the area and

the fact that their genetic status may be paramount to the outcome of impending legal battles between animal's rights groups and government agencies.

Materials & Methods

Samples and DNA Extraction

Samples from 404 canids (i.e. wolves and coyotes) were collected from across northern Ontario and the western Great Lakes states: northwestern Ontario, n = 92; northeastern Ontario, n = 97; Michigan, n = 114; Wisconsin, n = 48; Minnesota, n = 53. For this study these sampling locations collectively represent the western Great Lakes region (WGLR). These samples included whole tissue (n = 326), blood on FTA cards (n = 68), scat swabs (n = 8) and hairs (n = 2). DNA was extracted from samples using a DNeasy Blood and Tissue Kit (Qiagen Inc., Mississauga, ON). A Picogreen assay was used to quantify the concentration of DNA obtained from extraction for each sample.

Gender Determination

The following consensus primers were used to amplify 931 and 1087 base pair (bp) fragments of the X-chromosome and Y-chromosome respectively (Cathey *et al.* 1998).

LGL-331 5'-CAA ATC ATG CAA GGA TAG AC-3'

LGL-335 5'-AGA CCT GAT TCC AGA CAG TAC CA-3'

The Zfx/Zfy sexing fragments were amplified in a total reaction volume of 10-20 µl per tube using 5 ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 1.5 mM $MgCl_2$, 0.2 µM of primers LGL-331 and LGL-335, and 0.04 units of Taq polymerase (BRL). For blood, scat and hair samples 0.1 µg/µL of BSA was included in the reaction.

Products were amplified under the following conditions: 94°C for 5 min, 55°C for 30 sec, 72°C for 30 sec; 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, 30 cycles; 94°C for 30 sec, 55°C for 30 sec, 72°C for 10 min.

The following primer pairs were used to amplify 445 and 224 bp fragments of the X-chromosome (P2-3EZ and P1-5EZ: Aasen and Medrano 1990) and Y-chromosome (Y53-3C and Y53-3D: Fain and LeMay 1995) respectively.

P2-3EZ 5'-GCA CTT CTT TGG TAT CTG AGA AAG T-3'

P1-5EZ 5'-ATA ATC ACA TGG AGA GCC ACA AGC T-3'

Y53-3C 5'-CCC ATG AAC GCA TTC ATT GTG TGG-3'

Y53-3D 5'-ATT TTA GCC TTC CGA CGA GGT CGA TA-3'

The Zfx/Sry sexing fragments were amplified in a total reaction volume of 10-20 μl per tube using 5 ng of genomic DNA, 200 μM dNTPs, 1x amplification buffer, 2.5 mM $MgCl_2$, 0.2 μM of each primer, and 0.05 units of Taq polymerase (BRL). For blood, scat and hair samples 0.15 μg/μL of BSA was included in the reaction. Products were amplified under the following conditions: 95 for 1 min; 94 for 45 sec, 58 for 45 sec, 73 for 1 min, 35 cycles; 72 for 2 min.

The Zfx/Zfy primers were used for gender determination of scat samples because the Zfx/Sry primers can amplify non-target mammalian DNA that includes prey species of canids. The Zfx/Sry primers were used for gender determination of the tissue, blood and hair samples. For both primer sets the variation in fragment size between the X-chromosome and Y-chromosome results in males generating two bands of variable size and females generating two bands of equal size. Gender was determined for samples by visualization of banding patterns of amplified Zfx/Zfy or Zfx/Sry products on an

ethidium bromide stained agarose gel.

Mitochondrial DNA Control Region Sequencing

The following primers were used to amplify a 343-347 bp fragment of the control region of the mitochondrial DNA.

AB13279 5'-GAA GCT CTT GCT CCA CCA TC-3' (Pilgrim *et al.* 1998)

AB13280 5'-GGG CCC GGA GCG AGA AGA GGG AC-3' (Wilson *et al.* 2000)

The control region was amplified in a total reaction volume of 20 µl per tube using 5 ng of genomic DNA, 200 µM dNTPs, 1x amplification buffer, 1.5 mM $MgCl_2$, 0.2 µM of primers AB13279 and AB13280, and 0.05 units of Taq polymerase (BRL). For blood, scat and hair samples 0.1 µg/µL of BSA was included in the reaction. Products were amplified under the following conditions: 94°C for 5 min, 60°C for 30 sec, 72°C for 30 sec; 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 30 cycles; 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min. PCR products were cleaned using Exosap-IT (USB Corporation, Cleveland, OH) prior to sequencing on a MegaBACE 1000 (GE Healthcare).

Y-intron sequencing

The following Zfx/Zfy intron specific primers were used to amplify a 658 bp fragment of the final intron of the Zfy gene (Y-chromosome).

LGL-331 5'-CAA ATC ATG CAA GGA TAG AC-3' (Cathey *et al.* 1998)

Yint2-335 5'-GTC CAT TGG ATA ATT CTT TCC-3' (Shami 2002)

The Y-intron was amplified in a total reaction volume of 20 ul per tube using 5 ng of genomic DNA, 200 uM dNTPs, 1x amplification buffer, 1.5 mM $MgCl_2$, 0.2 μM of primers LGL-331 and Yint2-335, 0.1 μg/μL of BSA and 0.05 units of Taq polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min, 52°C for 30 sec, 72°C for 30 sec; 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, 35 cycles; 94°C for 30 sec, 52°C for 30 sec, 72°C for 10 min. PCR products were cleaned using Exosap-IT (USB Corporation, Cleveland, OH) prior to sequencing on a MegaBACE 1000 (GE Healthcare).

Microsatellite Genotyping

Amplification of twelve microsatellite loci was attempted for each sample (Ostrander *et al.* 1993 and 1995: cxx225, cxx200, cxx123, cxx377, cxx250, cxx204, cxx172, cxx109, cxx253, cxx442, cxx410, cxx147) in a total reaction volume of 15μl per tube using 5 ng of genomic DNA, 200 μM dNTPs, 1x amplification buffer, 1.5 mM $MgCl_2$, 0.2-0.3 μM of forward (labeled with fluorescent dye: 6FAM, NED or HEX) and reverse (unlabeled) primer, and 0.05 units of Taq polymerase (BRL). For blood, scat and hair samples 0.1-0.15 μg/μL of BSA was included in the reaction. Products were amplified under the following conditions: 94°C for 5 min; 94°C for 30 s, 56-58°C for 1 min, 72°C for 1 min, 30 cycles; 60°C for 45 min. PCR products were purified through ethanol precipitation prior to genotyping on a MegaBACE 1000 (GE Healthcare).

Genetic analysis

Mitochondrial DNA sequences were edited in Bioedit (Hall 1999) and assigned haplotypes, denoted as C(X), corresponding to the haplotypes described by Wilson *et al.* (2000 & 2003). Sequences were aligned using ClustalW multiple alignment in Bioedit, and then manually edited to achieve the best alignment, minimizing the number of variable sites (i.e. substitutions and gaps). The program MODELGENERATOR (Keane *et al.* 2006) was used to determine the most appropriate model of substitution for generating a phylogenetic tree. Based on the chosen alignment, the HKY model of substitution with a discrete gamma model of rate heterogeneity (alpha = 0.18) was selected as the most appropriate model for the data. The nucleotide base frequencies determined by MODELGENERATOR for the sequences were as follows: A = 0.27506, T = 0.30605, C = 0.28650, G = 0.13239. No transversions were observed among the sequences based on the chosen alignment.

A neighbor-joining tree was constructed using NEIGHBOR in PHYLIP (Felsenstein 2005), assuming a transition/transversion ratio of 15.47 (Vila *et al.* 1999), and using a HKY model with a gamma distribution (alpha = 0.18). A neighbor-joining tree was also constructed in MEGA version 4.0 (Tamura *et al.* 2007) using the Maximum Composite Likelihood (MCL) method with a gamma distribution (alpha = 0.18). The HKY model is not available in MEGA, however the MCL method is implemented under the Tamura-Nei substitution model in MEGA (Tamura *et al.* 2007), which was the fifth best model selected by MODELGENERATOR; therefore the MCL method was a suitable replacement for the HKY model. Tamura-Nei genetic distances have been used previously for analyses of canid mtDNA control region sequences (Andersone *et al.*

2002). A maximum parsimony tree was constructed using MEGA, including all sites and implementing a close-neighbor interchange with search level 3. For all trees support for branches was assessed based on 1000 bootstrap replicates and consensus trees were generated, rooted with a Golden Jackal sequence (C*anis aureus*, Genbank accession # AY289996). Both neighbor-joining trees and the maximum parsimony tree produced similar topologies.

Overall sequence diversity and the mean pair-wise sequence divergence within groups and between groups was calculated using the MCL method in MEGA with a gamma distribution (alpha = 0.18) and obtaining estimates of standard error from 1000 bootstrap replicates. Sequence comparisons incorporated pair-wise deletions, and the Golden Jackal sequence was omitted for the diversity and mean pair-wise sequence divergence calculations.

Y-intron sequences were edited and aligned in Bioedit (Hall 1999), and assigned haplotypes to correspond with the work of Shami (2002): CANInt1 = ancestral haplotype; CANInt2 = gray wolf haplotype; CANInt4 = eastern wolf haplotype.

Microsatellite alleles were scored in Genemarker (v1.7, SoftGenetics LLC, State College, PA) and unique genotypes were obtained for 371 samples: northwestern Ontario, n = 87; northeastern Ontario, n = 93; Michigan, n = 90; Wisconsin, n = 48; Minnesota, n = 53. Of the 371 genotyped individuals only eight samples were from low template DNA sources (i.e. scat or hair), thus genotyping error was assumed to be negligible overall since the samples consisted almost entirely of high template DNA sources (i.e. tissue and blood), reducing the likelihood of scoring errors due to allelic dropout. Homozygous allele scores were confirmed for two of the low template samples,

and five of the remaining low template samples for which homozygous alleles scores were not confirmed had heterozygous allele scores at eight or more of the twelve loci amplified, further reducing the potential for scoring errors due to allelic dropout. Additional samples (n = 256) from a previous study by Grewal (2001) were genotyped at the same twelve loci for inclusion in the genetic analyses to observe how the WGLR samples relate to canids from surrounding areas: Northwest Territories, n = 56; Manitoba, n = 36 (north of Duck Mountain National Park, n = 11; Duck Mountain National Park, n = 13; Riding Mountain National Park, n = 12); Quebec, n = 34 (western Quebec, n = 24; eastern Quebec, n = 10); Algonquin Provincial Park, n = 54 (L. Rutledge, unpublished data); Frontenac Axis, n = 52; Texas, n = 24.

The microsatellite genotype data from these 627 canids was analyzed using STRUCTURE (v2.2, Pritchard *et al.* 2000; Falush *et al.* 2003, 2007), which uses a Bayesian approach to infer the number of populations and estimates admixture proportions by assigning each multilocus genotype a probability of membership in each genetic cluster. The admixture model of STRUCTURE was run for K = 1 to K = 10 with five repetitions of 10^6 iterations following a burn-in period of 250,000 iterations for each K. The I-model (i.e. assumes independent allele frequencies among populations) and the F-model (i.e. allows for correlated allele frequencies among populations) were both implemented to compare results. For both models a separate alpha was inferred for each population to account for asymmetric admixture. The posterior probability (Ln P[D]) for a given K was computed by averaging the posterior probabilities across the five runs for that K. Based on a combination of criteria from Pritchard *et al.* (2000; maximal value of Ln P[D]) and Evanno *et al.* (2003; ΔK), and considering the overall individual ancestry

assignments and biological significance, the number of populations K was determined to be five for the I-model and seven for the F-model (Figure 2.1). The large ΔK value observed at K = 2 for both models likely reflects the hierarchical splitting of wolf-like from coyote-like canids (see Discussion), whereas the ΔK peak at K = 5 for the I-model (Figure 2.1) likely reflects the highest level of population subdivision, which is supported by the maximal value of Ln P(D) and high ancestry assignments of individuals to specific populations at K = 5. The determination of K for the F-model was less clear based on ΔK (Figure 2.1), however the maximal value of Ln P(D) at K = 7 and the high ancestry assignments of individuals to the two additional populations inferred by the F-model, relative to the I-model, support the population structure observed at K = 7 (see Discussion for additional interpretation of difference in K between models). For both models the data was run through STRUCTURE again for the selected value of K with ten repetitions and the individual admixture proportions (i.e. Q-values) were taken from the run having the highest posterior probability and lowest variance.

Tests of the assumptions inherent to STRUCTURE, i.e. Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE), were performed on the original geographic groups of samples, and on the groups of individuals highly assigned (Q>0.8) to the $K_I = 5$ and $K_F = 7$ populations inferred in STRUCTURE for the I-model and F-model respectively. Deviations from HWE were tested for all locus-population combinations and globally with an exact Hardy-Weinberg test using the Markov chain method (Guo and Thompson 1992) implemented in GENEPOP 4.0 (Rousset 2008). GENEPOP was also used to test pairwise LE among loci overall and within populations. The significance levels for HWE and LE tests were adjusted using the sequential

FIGURE 2.1

Bonferroni method to account for multiple tests on the same data (Rice 1989). Pairwise estimates of F_{ST} (Weir and Cockerham 1984) were computed in GENEPOP for all populations inferred by STRUCTURE (individuals with Q>0.8) for $K_I = 5$ and $K_F = 7$.

To supplement the results from STRUCTURE, a non-model based Factorial Correspondence Analysis (FCA) was performed on the microsatellite data for individual canids using GENETIX (v4.05, Belkhir *et al.* 2004). Two factorial components FC-1 and FC-2, which accounted for 5.75% and 3.23% of the total inertia respectively, were plotted to visualize the relative clustering of animals from the different sampling locations.

Results

Mitochondrial DNA haplotypes

A 223-229 bp (note C98 = 200 bp) informative region of the mtDNA control region was sequenced for 404 samples, and a previously identified diagnostic insertion/deletion was used to distinguish between Old World (OW) and New World (NW) mtDNA sequences (Pilgrim *et al.* 1998). Eighteen mtDNA haplotypes were observed in the study region: 13 were of NW origin (n = 260) and 5 were of OW origin (n = 144) (Table 2.1).

Phylogenetic analysis of mtDNA sequences revealed three haplotype groupings similar to those observed by Wilson *et al.* (2000 & 2003), corresponding to gray wolves, eastern wolves and coyotes (Figure 2.2). Overall sequence diversity was 0.118 ±0.044 (S.E.), and average pair-wise sequence divergence within groups was 2.0 ±0.8% (range 0.5─3.3%) for gray wolves, 1.5 ±0.8% for eastern wolves, and 2.4 ±0.9% (range 0.5─5.7%) for coyotes. Average pair-wise sequence divergence between groups was

TABLE 2.1

FIGURE 2.2

14.8 ±6.9% (range 13.2─16.6%) for gray wolves and eastern wolves, 25.4 ±11.7% (range 17.4─34.3%) for gray wolves and coyotes, and 4.7 ±1.9% (range 2.6─7.4%) for eastern wolves and coyotes.

The distribution of haplotypes showed extensive overlap of OW and NW haplotypes throughout the study region (Figure 2.3). The most prevalent haplotypes were C3 (n = 70), C13 (n = 121), and C22 (n = 114), which were collectively observed in ~75% of the animals sampled spanning the majority of the study region (Table 2.1, Figure 2.3). The other observed haplotypes occurred in lower frequency (i.e. n<25) across the study region, although haplotype C14 was the predominant NW haplotype in northeastern Ontario (Figure 2.3).

Y-intron haplotypes

Genetic determination of gender revealed 161 females and 211 males (Table 2.1), and gender was not successfully determined for the remaining 32 samples. Y-intron sequences were obtained for 91% of genetically identified male canids. A 463 bp fragment was sequenced that incorporated the variable sites identified by Shami (2002), from which three unique haplotypes were observed: ancestral, n = 13; gray wolf, n = 106; eastern wolf, n = 74 (Table 2.1). The gray wolf and eastern wolf Y-intron haplotypes were far more prevalent than the ancestral haplotype. The coyote Y-intron haplotype (i.e. CANInt3) observed by Shami (2002) was not observed in any animals in the study region. The distribution of Y-intron haplotypes in the study area showed extensive overlap of the eastern wolf and gray wolf Y-intron haplotypes (Figure 2.4). The gray wolf Y-intron haplotype was predominant in northeastern Ontario, however, the eastern wolf

FIGURE 2.3

FIGURE 2.4

Y-intron haplotype was still present in this region (Figure 2.4). The ancestral Y-intron haplotype was not observed in the northern portions of the study region (Figure 2.4).

Microsatellite genotypes

The microsatellite loci had on average 9.5 ±3.4 (S.D.) alleles per locus (range 5 to 16). The geographic sample groups showed overall deviations from HWE at seven loci and deviations from LE for twelve pairs of loci. Several specific geographic sample groups showed deviations from HWE at one or more loci and deviations from LE for one or more pairs of loci. The populations inferred by STRUCTURE (individuals with Q>0.8) were in overall HWE for $K_I = 5$ and $K_F = 7$, and all loci for all populations were individually in HWE. The STRUCTURE inferred populations were in overall LE across all pairs of loci, however, for $K_I = 5$ three populations showed deviations from LE for one pair of loci each, and for $K_F = 7$ one population showed deviation from LE for one pair of loci. These results suggest that the deviations from HWE and LE present in the geographic sample groups were appropriately modeled by STRUCTURE to generate the genetically inferred populations, which conform to HWE and LE.

The I-model in STRUCTURE inferred five genetic populations (Figure 2.5): P_I1 = Texas; P_I2 = Frontenac Axis; P_I3 = Algonquin Park; P_I4 = Manitoba, Minnesota, Wisconsin, Michigan, northwestern/northeastern Ontario and Quebec; P_I5 = Northwest Territories. The F-model in STRUCTURE inferred seven genetic populations (Figure 2.5): P_F1 = Texas; P_F2 = Frontenac Axis; P_F3 = Algonquin Park; P_F4 = Minnesota, Wisconsin, Michigan and northwestern Ontario; P_F5 = northeastern Ontario and Quebec; P_F6 = Manitoba; P_F7 = Northwest Territories.

FIGURE 2.5

The difference in clustering between the I-model and F-model is that P_I4 inferred under the I-model splits into P_F4, P_F5 and P_F6 under the F-model. Multimodal probabilities were encountered for some values of K for both the I-model (at K = 4) and the F-model (at K = 4, K = 5 and K = 6), resulting in variable clustering of groups for a given value of K, i.e. certain groups sorted out alternatively between two sequential values of K. However the I-model was more stable than the F-model, and showed consistent clustering and individual ancestry assignments among runs at $K_I = 5$, the value for which support was strongest based on the criteria of Pritchard *et al.* (2000) and Evanno *et al.* (2003). The F-model showed more inconsistency in clustering of groups than the I-model, specifically in how groups sorted out between $K_F = 4$ and $K_F = 6$. Although, the F-model showed some variation in individual ancestry assignments among runs at $K_F = 7$, it consistently identified the presence of the P_F5 and P_F6 clusters, with both having notable numbers of highly assigned individuals. The highest posterior probability occurred at $K_F = 7$ under the F-model, suggesting that the population structure observed at $K_F = 7$ exists, but it is likely complex. The F-model is able to detect more subtle population structuring but may tend to overestimate K (Falush *et al.* 2003). Given that $K_I = 5$ was more stable than $K_F = 7$, and P_I4 included the extra two populations inferred under the F-model (P_F5 and P_F6), it was determined that $K_I = 5$ provided a more reliable and conservative broad taxonomic view of the population structure. Therefore, the $K_I = 5$ admixture proportions of individual canids were deemed appropriate for relating to the mtDNA and Y-intron haplotype content of the animals.

Pairwise F_{ST} values revealed the genetic differentiation between the populations inferred by STRUCTURE, indicating a relatively low genetic differentiation between P_I1

and P_I2 (same for P_F1 and P_F2), and a relatively large genetic differentiation between P_I1 and P_I5 (same for P_F1 and P_F7) (Table 2.2). Notably, there was relatively low genetic differentiation between P_F4 and P_F5 (Table 2.2).

The FCA revealed roughly five distinct clusters, represented by the Northwest Territories, Algonquin Park, Texas and Frontenac Axis sample groups, and one large cluster of the western Great Lakes states, northwestern/northeastern Ontario, Manitoba, and Quebec sample groups (Figure 2.6). The northeastern Ontario and Manitoba sample groups clustered notably closer to the Northwest Territories samples and showed some deviation from the core cluster of the western Great Lakes states and northwestern Ontario groups (Figure 2.6). Several individuals from the western Great Lakes states and northwestern Ontario sample groups were observed clustering relatively intermediate to the Frontenac Axis and Texas samples groups, and several individuals from northeastern Ontario were observed clustering closely with the Frontenac Axis samples (Figure 2.6).

Combined genetic data

The ancestral Y-intron haplotype was only observed in animals with a NW mtDNA haplotype (Table 2.1) and high assignment to either P_I1 or P_I2, with the exception of one animal that had a mixed assignment to P_I3 and P_I4. The gray wolf and eastern wolf Y-intron haplotypes occurred in highest frequency in animals having the C3, C13 or C22 mtDNA haplotype, and the gray wolf Y-intron haplotype was also observed in twelve animals with mtDNA haplotype C14 in northeastern Ontario (Table 2.1). The majority of animals having a coyote mtDNA haplotype (note that C13 and C14 are interpreted as

TABLE 2.2

FIGURE 2.6

haplotypes derived primarily from *C. lycaon* in the study region, see Discussion) had high assignment to the P_I1 or P_I2 cluster, with the exception of haplotype C9, which despite occurring predominantly in animals with high assignment to P_I1 or P_I2, was also observed in several animals with notable ancestry to one or more of the other three genetic clusters. The animals with high assignment to the P_I4 cluster had a mixture of NW and OW mtDNA haplotypes with either a gray wolf or eastern wolf Y-intron haplotype. The majority of animals with high assignment to the P_I4 cluster had the C3, C13 or C22 mtDNA haplotype, which were the most prevalent haplotypes as mentioned above, and none of these haplotypes were observed in animals with high assignment to P_I1 or P_I2. The combination of a coyote mtDNA haplotype and a gray wolf Y-intron haplotype was rare, being observed in only six animals (Table 2.1, note that C13 and C14 are interpreted as haplotypes derived primarily from *C. lycaon* in the study region, see Discussion). The OW mtDNA haplotypes occurred exclusively in animals having either the gray wolf or eastern wolf Y-intron haplotype (Table 2.1). Also, the OW mtDNA haplotypes occurred exclusively in animals having high assignment to P_I4 or P_I5, or mixed assignment to P_I3, P_I4, and P_I5, with the exception of one individual that had a mixed assignment to P_I1, P_I2 and P_I4. The gray wolf Y-intron haplotype occurred predominantly in animals having high assignment to P_I4 or P_I5, with a few animals having notable assignment to P_I3, and only two animals having high assignment to P_I1 or P_I2. The eastern wolf Y-intron haplotype occurred predominantly in animals having high assignment to P_I4, however, there were several animals with high assignment to P_I2 and one animal that had mixed ancestry to P_I3, P_I4 and P_I5.

The overall notable trends for the combined genetic data were: 1) coyote mtDNA was observed in animals with high assignment to P_I1 or P_I2; 2) eastern wolf or gray wolf mtDNA was observed predominantly in animals with eastern wolf or gray wolf Y-intron and high assignment to P_I4; and 3) rare co-occurrence of gray wolf Y-intron and coyote mtDNA haplotypes.

Discussion

Canid samples (n = 404) from the WGLR were genetically characterized using three genetic markers, and compared to wolves and coyotes from other regions in North America. Based on mtDNA and Y-intron data from this study and others (e.g. Grewal 2001; Shami 2002), and considering the overall relative clustering of groups based on the microsatellite genotypes, the populations inferred by STRUCTURE can be assigned a species or hybrid designation (P_In = I-model; P_Fn = F-model): P_I1 and P_F1 = *C. latrans* (Texas); P_I2 and P_F2 = *C. latrans-lycaon* hybrids (Frontenac Axis); P_I3 and P_F3 = *C. lycaon* (Algonquin); P_I4 = *C. lupus-lycaon* hybrids (Manitoba, Minnesota, Wisconsin, Michigan, northwestern/northeastern Ontario and Quebec); P_I5 and P_F7 = *C. lupus* (Northwest Territories). The animals belonging to P_F4 (Minnesota, Wisconsin, Michigan and northwestern Ontario), P_F5 (northeastern Ontario and Quebec) and P_F6 (Manitoba) all represent *C. lupus-lycaon* hybrids, and are collectively representative of the animals comprising P_I4 (Figure 2.5).

Haplotype C13 occurred in high frequency in the western Great Lakes states (n = 88 of 215; 41%) and northwestern Ontario (n = 25 of 92; 27%), and haplotype C14 occurred in relatively high frequency in northeastern Ontario (n = 23 of 97; 24%) (Figure

2.3). Haplotypes C13 and C14 were observed predominantly in *C. lupus-lycaon* hybrids and not coyotes, therefore they were interpreted to represent mtDNA haplotypes derived primarily from *C. lycaon* in the study region, even though they clustered with coyote sequences (Figure 2.2). Mitochondrial haplotypes C13 and C14 have both been observed in eastern coyotes from southeastern Ontario (Grewal *et al.* 2004). Haplotype C14 has also been observed in non-hybridizing coyotes in Nebraska (C14 is identical to haplotype la033 in Hailer and Leonard 2008), however, haplotype C13 has not been observed in non-hybridizing populations of coyotes (e.g. Wilson *et al.* 2003; Hailer and Leonard 2008; C13 not observed in Saskatchewan coyotes, data not shown). Therefore haplotype C13 is suggested as being of eastern wolf origin through the introgressive hybridization of a coyote haplotype and subsequent divergence to become eastern wolf specific (see Chapter 3). The presence of haplotype C14 in non-hybridizing populations of coyotes indicates that it is a *C. latrans* haplotype and suggests the introgression of haplotype C14 into *C. lupus-lycaon* hybrids through previous introgression into eastern wolves. Thus, haplotypes C13 and C14 are both interpreted as being derived from *C. lycaon* in the study region, but through different mechanisms. These findings illustrate the extensive hybridization that has occurred between eastern wolves and coyotes, which has resulted in haplotypes of both species being observed in various hybrids, i.e. eastern wolf haplotypes occur in eastern coyotes and putative coyote haplotypes occur in *C. lupus-lycaon* hybrids.

The ancestral Y-intron haplotype was observed exclusively in animals with coyote mtDNA and having high assignment to P_I1 or P_I2, with the exception of one animal having a C14 mtDNA haplotype and mixed assignment to P_I3 and P_I4. Therefore,

based on the samples analyzed, the ancestral Y-intron haplotype is interpreted to be generally indicative of a coyote paternal contribution in the study region, where the coyote specific Y-intron haplotype has not been observed (i.e. CANInt3 from Shami 2002). However, it is recognized that the ancestral Y-intron haplotype does occur in wolves in North America (Shami 2002), and thus does not exclusively represent a coyote paternal contribution.

The results of the FCA were concordant with the results from STRUCTURE, revealing approximately five genetic clusters. Given that predominantly gray wolf and eastern wolf mtDNA and Y-intron haplotypes occurred in P_I4 animals, this population was concluded to represent animals containing genetic material derived from both gray wolves and eastern wolves. Therefore, P_I4 represents a population of *C. lupus-lycaon* hybrids that extends from the western Great Lakes states into northwestern Ontario, and also into Manitoba, northeastern Ontario and Quebec (Figure 2.5). Coyotes from the western Great Lakes states and northern Ontario showed high assignment to P_I1 or P_I2, and this is confirmed by the FCA (Figure 2.6). Only one coyote mtDNA haplotype (C9) and no ancestral Y-intron haplotypes were observed in P_I4 animals, and there was a very low level of admixture between P_I4 and either P_I1 or P_I2 (Figure 2.5). These findings indicate that *C. lupus-lycaon* hybrids do not breed with coyotes.

The splitting of Manitoba, and northeastern Ontario and Quebec from P_I4 under the F-model in STRUCTURE is reflected in the results of the FCA, which showed samples from these groups deviating noticeably from the core cluster of the western Great Lakes states and northwestern Ontario samples (Figure 2.6). The Manitoba (P_F6), and northeastern Ontario and Quebec (P_F5) populations still represent *C. lupus-lycaon*

hybrids based on mtDNA (Wilson *et al.* 2003; Grewal *et al.* 2004) and Y-intron haplotypes (i.e. in northeastern Ontario). There was a notable difference in mtDNA and Y-intron haplotype composition between northeastern Ontario wolves and those from the western Great Lakes states and northwestern Ontario. The gray wolf Y-intron haplotype occurred in much higher frequency than the eastern wolf Y-intron haplotype in northeastern Ontario compared to in the west (Figure 2.4). Also, mtDNA haplotype C14 was observed in relatively high frequency in northeastern Ontario compared to C13, which was the predominant haplotype to the west (Figure 2.3). These differences in haplotype composition suggest that the F-model clustering of northeastern Ontario as separate from P_I4 represents real population differentiation. The separate clustering of Manitoba under the F-model is also supported by the greater proportion of gray wolf mtDNA haplotypes observed relative to eastern wolf haplotypes in Manitoba (Wilson *et al.* 2003) compared to the WGLR, suggesting Manitoba *C. lupus-lycaon* hybrids are proportionally more gray wolf, although the pairwise F_{ST} values do not support this (Table 2.2). Regardless, the results are suggestive of an east to west genetic cline of *C. lupus-lycaon* hybrids in northeastern North America. Collection of more samples from northern Ontario, Quebec and Manitoba is necessary to further assess the genetic differentiation between the various *C. lupus-lycaon* populations. The analysis of linked and unlinked loci in STRUCTURE, implementing the linkage model, is recommended and could provide information on the time since hybridization for the *C. lupus-lycaon* populations, which may indicate a possible east-west difference in time since hybridization between eastern wolves and gray wolves if it exists. The western *C. lupus-lycaon* hybrids may represent a longer-standing hybrid than the eastern *C. lupus-lycaon*

hybrids (see Chapter 3), however, the analysis of historic samples from both regions is likely also required for a conclusive assessment to be made.

The pairwise estimates of F_{ST} revealed a relatively low genetic differentiation between Texas coyotes and Frontenac Axis eastern coyotes. The eastern wolves of Algonquin Park exhibited least genetic differentiation to Frontenac Axis eastern coyotes, which reflects their geographic proximity and sharing of eastern wolf and coyote genetic material (e.g. Grewal *et al.* 2004). Genetic differentiation between Northwest Territories gray wolves and Texas coyotes was relatively high, as was expected given their relatively distant evolutionary relationship. The *C. lupus-lycaon* hybrids from the western Great Lakes states and northwestern Ontario showed relatively low genetic differentiation to the *C. lupus-lycaon* hybrids from northeastern Ontario and Quebec, generally consistent with the STRUCTURE and FCA results.

A notable number of eastern coyotes were observed in northeastern Ontario, but primarily in the southern portion of this region, indicating that the range of eastern coyotes extends into northeastern Ontario. The occurrence of several animals with high assignment to P_I2 was also observed in the western Great Lakes states; however this does not necessarily suggest that eastern coyotes are present in this region. This is reflected in the FCA (Figure 2.6), in which samples from the western Great Lakes states clustered at the fringe of the Frontenac Axis group, somewhat intermediate to the Texas and Frontenac Axis samples, and among the samples from northwestern Ontario that had high assignment to P_I1 instead of P_I2. This contrasts with the animals from northeastern Ontario that had high assignment to P_I2, which generally clustered at the core of the Frontenac Axis group in the FCA (Figure 2.6) and likely represent eastern coyotes similar

to those found in southeastern Ontario. These results suggest that the coyote groups used in the analysis may not be representative of the coyotes from the western Great Lakes states and northwestern Ontario, rather they may provide the most closely related populations for ancestry assignment of WGLR coyotes. Non-hybridizing coyote populations from closer regions may provide a more accurate assessment of the distribution and genetic ancestry of coyotes from the WGLR to confirm the presence, or more likely the absence of eastern coyotes (i.e. *Canis latrans X lycaon*) in the WGLR. Regardless of this valid concern, the coyote groups included in the analysis did effectively identify coyote-like animals as different from the wolves of P_I4 and the other two wolf populations. The multiple streams of genetic data collectively suggest that wolf-coyote hybridization in the WGLR is rare, however, the observation of a few individuals that were classified as having mixed wolf-coyote ancestry suggests that *C. lupus-lycaon* hybrids are potentially capable of breeding with sympatric coyotes. A larger sampling of putative pure coyotes from the WGLR is required to further assess their relationship to the *C. lupus-lycaon* hybrids.

The finding that *C. lupus-lycaon* hybrids occupy the WGLR and do not breed with sympatric coyotes contrasts with previous studies that suggested *lupus/latrans* hybridization was prevalent in this region (e.g. Lehman *et al.* 1991). However, the data from some of these previous studies can be interpreted to support to the findings of this study. For instance, Lehman *et al.* (1991) reported the observation of "coywolf" (i.e. NW) mtDNA genotypes in wolves from northeastern Minnesota and northwestern Ontario, suggesting that the combination of NW and OW mtDNA was indicative of *lupus/latrans* hybridization. However, when the eastern wolf is considered as a NW

replacement for the coyote given their data, the results are consistent with *lupus/lycaon* hybridization. Specifically, Lehman *et al.* (1991) reported "coywolf" mtDNA genotypes W7 and W9 were observed in high frequency in hybridizing wolves from northeastern Minnesota and northwestern Ontario, which based on relative frequencies are probably analogous to *C. lycaon* mtDNA haplotypes C13 and C3 observed in this study. The similarity of eastern wolf and coyote mtDNA haplotypes previously led researchers to mischaracterize hybridization when the eastern wolf is not integrated into the interpretation of the genetic data. In the absence of supporting data from nuclear DNA the differences between hybridizing wolves to the west and east of the Great Lakes were not properly assessed.

The finding that *C. lupus-lycaon* hybrids occupy the WGLR and do not breed with sympatric coyotes based on genetic analyses is supported by morphological analyses of animals from this region (e.g. Nowak 1995 & 2003). There is no evidence to support wolf-coyote hybridization extending beyond southeastern Ontario and Quebec based on morphology, ecology or behaviour, and wolves of Minnesota and northwestern Ontario are fully wolf-like in those respects (Nowak 2003). A review of wolf taxonomy based on morphological studies supported wolf (*C. lupus lycaon*) populations from Minnesota being more closely related to gray wolves (*C. lupus nubilus*) than to wolf (*C. lupus lycaon*) populations from Quebec and southeastern Ontario (see Nowak 1995). The review supported the suggestion of Kolenosky and Standfield (1975) that northwestern Ontario populations are closely related to Minnesota populations and are separated by a sub-specific line from populations in southeastern Ontario, with larger wolves to the northwest and smaller wolves to the southeast in Ontario. The classification of canids in

the Great Lakes region based on morphology (Nowak 1995) is congruent with the classification based on genetics from this study.

The interpretation of *Canis* hybridization in northeastern North America based on current data should be that wolves in the WGLR represent *C. lupus-lycaon* hybrids that do not breed with sympatric coyotes, whereas wolves in the southeastern Great Lakes region represent *C. latrans-lycaon* hybrids (i.e. eastern coyotes). Algonquin Park, located in central Ontario, appears to represent a putatively purer eastern wolf population, however, introgression of coyote and gray wolf genetic material has occurred (Grewal *et al.* 2004). The degree to which *C. lupus-lycaon* hybrids may breed with eastern coyotes is presently unclear, however data from this study suggests that it is not common. The presence of the same coyote mtDNA haplotypes in both eastern coyotes and *C. lupus-lycaon* hybrids (i.e. haplotype C14) would suggest that these two different types of hybrids do interbreed, however, the nuclear DNA profiles indicate that coyote genes are not prevalent in the *C. lupus-lycaon* hybrids, even those containing coyote mtDNA haplotypes. This supports the introgression of putative coyote mtDNA into *C. lupus-lycaon* hybrids through previous introgression into eastern wolves (i.e. haplotype C14). Further sampling of central Ontario, near Algonquin Park, where the ranges of *C. lupus-lycaon* hybrids and eastern coyotes likely converge is required to further assess this issue, and is important for determining the extent of a potential three species hybrid complex.

The genetic composition of extant wolves in the WGLR is consistent with Wisconsin and Michigan being recolonized by wolves coming from Minnesota and northwestern Ontario, as indicated by the large homogeneous wolf population that currently occupies the region. The population structure of WGLR wolves suggests high

levels of gene flow and connectivity, and accordingly conservation and management strategies should focus on conserving this genetic connectivity. The combination of eastern wolf and gray wolf genetic material in WGLR wolves should provide adaptive evolutionary potential to changing environmental factors such as prey, climate and habitat. Although the wolf population is presently not breeding with sympatric coyotes in the WGLR, it is uncertain how environmental changes in the future may affect the hybridization dynamics of canids in this region. However, based on the fact that WGLR wolves occurred at low densities for decades sympatric with coyotes prior to their recovery, and they presently show no notable evidence of coyote introgression, the threat of wolf-coyote hybridization does not seem significant. Regardless, genetic monitoring of WGLR wolves should continue if potential hybridization with coyotes is considered a major concern for their conservation and management, given that changes in the landscape and climate may impact the dynamics of *Canis* populations in the future.

The findings of this study indicate that wolf recovery efforts have been successful in restoring a large, genetically homogeneous wolf population to the WGLR, and state management plans should focus on maintaining adequate numbers of wolves to ensure their long-term persistence in the region, rather than worrying about the genetic ancestry of, and relationships among, canids in this region.

Chapter 3: Genetic analysis of historic western Great Lakes region wolf samples reveals early *Canis lupus/lycaon* hybridization

Abstract

The genetic status of wolves in the western Great Lakes region has received increased attention following the decision to remove them from protection under the U.S. Endangered Species Act. A recent study of mitochondrial DNA suggested that the recovered wolf population is not genetically representative of the historic population. Microsatellite genotype data of three historic samples is presented and compared to extant populations, and published genetic data are interpreted to show that the pre-recovery population was admixed over a century ago by eastern wolf (*Canis lycaon*) and gray wolf (*C. lupus*) hybridization. The DNA profiles of the historic samples are similar to those of extant animals in the region, suggesting that the current Great Lakes wolves are representative of the historic population.

Introduction

The ongoing debate over the evolutionary history and genetics of *Canis* populations in northeastern North America has become of immediate relevance to the conservation and management of wolves given the recent U.S. federal de-listing of the Western Great Lakes Distinct Population Segment (FWS 2007). Various studies over the last two decades have focused on the genetic composition of *Canis* in the Great Lakes Region (GLR), and genetic data have shown that wolves in this region contain genetic material of Old World (OW) and New World (NW) evolved species (Lehman *et al.* 1991), yet much uncertainty remains about their relationship with other populations.

Recently, Leonard and Wayne (2008) reported on mitochondrial DNA (mtDNA) analyses of historic GLR wolves, suggesting that pre-recovery wolves were dominated by haplotypes distinct from gray wolves (*Canis lupus*) and western coyotes (*C. latrans*) that they propose are from an endemic North American wolf referred to as the "Great Lakes wolf". They interpreted the current population to be admixed, deriving primarily from *lupus/latrans* hybridization, with minor contributions from the "Great Lakes wolf", and concluded that recently de-listed GLR wolves are not genetically representative of the pre-recovery population. Their interpretation fails to recognize extensive genetic data on the NW evolved eastern wolf (*C. lycaon*), the mtDNA sequences of which are close to those of *C. latrans* (Wilson *et al.* 2000 & 2003). The eastern wolf has been shown to be a distinct species (Wilson *et al.* 2000) that is capable of hybridizing with both coyotes and gray wolves across its range (see Kyle *et al.* 2006), acting as the conduit of *Canis* hybridization in northeastern North America. Some researchers do not agree with the suggestion that the current GLR population contains animals derived from *lupus/latrans* hybridization, as these sympatric species do not hybridize in western North America (see Kyle *et al.* 2006).

Nuclear microsatellite and mtDNA data from three pre-recovery samples from the western GLR are presented, and mtDNA haplotypes from Leonard and Wayne (2008) are compared to those reported by Wilson *et al.* (2000 & 2003) as evidence that the present and pre-recovery wolf populations in the western GLR are genetically similar and are derived from *lupus/lycaon* hybridization.

Materials and Methods

Samples and DNA extraction

DNA was extracted from three historic C*anis* samples provided by the University of Wisconsin Zoological Museum (Table 3.1) using a DNeasy Blood & Tissue Kit (Qiagen Inc., Mississauga, ON) in a dedicated ancient DNA laboratory.

Table 3.1: Historic *Canis* sample information.

museum catalog#	sex	state	country	date
8626	male	Minnesota	Itasca	Spring 1900
8627	male	Minnesota	Itasca	February 1899
11856	--	Wisconsin	Ashland	Winter 1907/08

Mitochondrial Control Region Sequencing

A 343-347 base-pair (bp) fragment of the mtDNA control region was amplified using the primers described in Chapter 2 and under similar conditions with 0.1 μg/μL of BSA included in the reaction. Contamination was monitored during extraction and PCR using negative controls. PCR products were cleaned with Exosap-IT (USB Corporation, Cleveland, OH) prior to sequencing on a MegaBACE 1000 (GE Healthcare). The sequence of sample 11856 was confirmed from an independent amplification. Sequences were edited, aligned and compared to known haplotypes in Bioedit (Hall 1999). Refer to Chapter 2 for a description of the sequences.

Microsatellite Genotyping

Amplification of twelve nuclear microsatellite loci was attempted for each sample (refer to Chapter 2), and homozygous genotypes were confirmed by repeated amplification. Two samples were genotyped at twelve loci and the remaining sample at eight loci.

Genetic analysis

Alleles were scored in Genemarker (v1.7, SoftGenetics LLC, State College, PA) and the data were analyzed using STRUCTURE (v2.2, Pritchard *et al.* 2000; Falush *et al.* 2003, 2007) including the following samples genotyped at the same twelve loci as the historic samples: Northwest Territories (n = 56); Manitoba (n = 36); Minnesota (n = 53); Wisconsin (n = 48); Michigan (n = 90); Northwestern Ontario (n = 87); Northeastern Ontario (n = 93); Quebec (n = 34); Algonquin Provincial Park (n = 54); Frontenac Axis (n = 52); Texas (n = 24). Based on a previously described STRUCTURE analysis (see Chapter 2) the number of populations K for the data set was determined to be five (based on I-model). The admixture model of STRUCTURE was run at K = 5 with ten repetitions of 10^6 iterations following a burn-in period of 250,000 iterations, assigning each historic sample a proportional membership to each of the five inferred genetic clusters. All ten STRUCTURE runs had similar posterior probabilities (Ln P[D]) and variances; therefore the proportional memberships of the historic samples were taken from the run having the lowest variance and highest posterior probability.

To supplement the results from STRUCTURE, a non-model based Factorial Correspondence Analysis (FCA) was performed on the microsatellite data for individual canids using GENETIX (v4.05, Belkhir *et al.* 2004). Two factorial components FC-1 and FC-2, which accounted for 5.74% and 3.23% of the total inertia respectively, were plotted to visualize the clustering of the historic samples in relation to the other sample groups.

Results

The informative variable ~230 bp region of the "Great Lakes wolf" mtDNA control region haplotypes within Leonard and Wayne (2008), denoted as GL(X), was compared to sequences described in Wilson *et al.* (2000 & 2003), denoted as C(X). As previously identified by Leonard and Wayne (2008), haplotype GL1 was identical to *C. lycaon* haplotype C1. However, other similarities were observed among haplotypes from both studies (Table 3.2), including two GL(X) haplotypes identical to *C. lycaon* haplotype C3. It is of interest that three GL(X) haplotypes were identical to a coyote-clustering sequence, haplotype C13, which has not been found in extant non-hybridizing coyote populations but is present throughout the distribution of *C. lycaon* (Wilson *et al.* 2003; Grewal *et al.* 2004).

The three historic samples were sequenced at the mtDNA control region (294-322 bp) and assigned haplotypes based on the ~230 bp region (Table 3.3). The two haplotypes observed in the three samples, C13 (n = 2) and C1 (n = 1), were identical to haplotypes found by Leonard and Wayne (2008) (Table 3.2).

Based on the genotypes at the microsatellite loci five populations were identified by STRUCTURE: P1 = Texas (western coyotes); P2 = Frontenac Axis (eastern coyotes); P3 = Algonquin (eastern wolves); P4= Manitoba, Minnesota, Wisconsin, Michigan, Quebec and northwestern/northeastern Ontario (eastern/gray wolves); P5 = Northwest Territories (gray wolves). The admixture proportions of the three historic samples revealed their highest proportional memberships were to P4, and one sample had 25% assignment to P3 (Table 3.3). The individual-based FCA clustered the historic samples

TABLE 3.2

amongst samples from the Manitoba, western Great Lakes states, Quebec and northwestern/northeastern Ontario groups (Figure 3.1).

Table 3.3: Mitochondrial DNA haplotypes and admixture proportions of historic *Canis* samples. Populations inferred by STRUCTURE: P1 = Texas (western coyotes); P2 = Frontenac Axis (eastern coyotes); P3 = Algonquin (eastern wolves); P4= Manitoba, Minnesota, Wisconsin, Michigan, Quebec and northwestern/northeastern Ontario (eastern/gray wolves); P5 = Northwest Territories (gray wolves).

museum catalog#	haplotype	# loci	admixture proportions				
			P1	P2	P3	P4	P5
8626	C13	12	0.009	0.013	0.042	0.922	0.015
8627	C13	8	0.013	0.012	0.250	0.673	0.053
11856	C1	12	0.011	0.015	0.097	0.814	0.063

Discussion

The genetic analyses revealed that the three historic samples from the western GLR have a mixed ancestry deriving primarily from sample groups representing eastern wolves and gray wolves (i.e. P4). The historic samples did not cluster significantly with either of the two groups composed of coyote-like animals (i.e. P1 and P2). The results of the FCA were concordant with the results from STRUCTURE with the historic samples clustering with wolves and not coyotes. Both OW and NW mtDNA haplotypes occur in P3 (Grewal *et al.* 2004) and P4 (see Chapter 2; Wilson *et al.* 2008), whereas only OW haplotypes occur in P5 (Wilson *et al.* 2003) and only NW haplotypes occur in P1 (Wilson *et al.* 2003) and P2 (Grewal *et al.* 2004) (Figure 3.1). Given that OW and NW haplotypes occur in the group for which the historic samples had their highest proportional memberships (i.e. P4), the historic samples were concluded to represent animals containing genetic material derived from both gray wolves (OW) and eastern wolves (NW), and not coyotes.

FIGURE 3.1

The occurrence of haplotype C13 in two historic samples that clustered with non-coyote groups based on nuclear microsatellite data (Table 3.3) supports the interpretation that C13 is a *C. lycaon* haplotype, as does its apparent absence from extant coyote populations in regions with no evidence of wolf-coyote hybridization, such as Texas (Wilson *et al.* 2003) and Nebraska (Hailer and Leonard 2008). The occurrence of haplotype C13 in wolves 100 years ago is likely the result of one of three possible scenarios: 1) C13 evolved in the common ancestor of coyotes and eastern wolves and was perpetuated in both species when they diverged (i.e. incomplete lineage sorting); 2) *lycaon/latrans* hybridization occurred earlier, i.e. pre-European settlement, whereby C13 was introgressed into *C. lycaon* and subsequently lost from the source *C. latrans* population; and 3) an ancestral coyote haplotype was introgressed into the *C. lycaon* lineage during the Pleistocene or sometime prior to European settlement and subsequently diverged to become eastern wolf specific. The latter scenario would explain why C13 clusters closer to coyote sequences than eastern wolf sequences (Wilson *et al.* 2003). The loss of a mtDNA haplotype from a source *C. latrans* population seems unlikely given the rapid population expansion of the species and the apparent absence of C13 from non-hybridizing coyote populations. The divergence of haplotype C13 from the eastern wolf clade and its absence in western coyote populations (Wilson *et al.* 2003) supports C13 as being of eastern wolf origin through introgressive hybridization and subsequent divergence, and not incomplete lineage sorting.

The GL(X) haplotypes that were identical to haplotypes C1, C3, and C13 (Table 3.2) occurred in samples from the western Great Lakes states (Leonard and Wayne 2008), further supporting the presence of *C. lycaon* genetic material in animals in this region.

The ability of the eastern wolf to hybridize with both coyotes and gray wolves complicates species assignments based on mitochondrial sequences and leads to questions concerning their validity, because the possibility exists of NW haplotypes occurring in *C. lupus-lycaon* hybrids and OW haplotypes occurring in *C. latrans-lycaon* hybrids. This issue has important ramifications for previous taxonomic interpretations based solely on mtDNA.

The DNA profiles presented here indicate that the pre-recovery western GLR wolf population was probably composed of *C. lupus-lycaon* hybrids, suggesting that eastern wolves and gray wolves hybridized historically (i.e. >100ya). To date no *C. lupus* mtDNA has been observed in pre-recovery western GLR samples, however, based on the nuclear microsatellite data, these animals are genetically similar to the present animals which have both gray wolf and eastern wolf mtDNA haplotypes. Limited sampling has likely failed to resolve the presence of *C. lupus* mtDNA in the western GLR during pre-recovery times (Nowak 2002), accepting that it may have been present at a lower frequency than in the current population. Several factors may have contributed to the observed absence or suspected lower abundance of gray wolf haplotypes in pre-recovery western GLR wolves: 1) population bottleneck; 2) genetic drift; and 3) sex-biased *lupus/lycaon* hybridization.

A probable scenario is that re-colonizing wolves originating from Minnesota, Manitoba and northwestern Ontario, containing *C. lupus* and *C. lycaon* mtDNA haplotypes, moved east into Wisconsin and continued into Michigan's Upper Peninsula. This may have resulted in current wolf populations exhibiting *C. lupus* mtDNA in higher frequency than in pre-recovery wolves from the western GLR. Previous research supports

the presence of both *C. lycaon* and *C. lupus* haplotypes in Manitoba and northwestern Ontario (Wilson *et al.* 2003; see Chapter 2), which represents a potential and likely source of immigrants for the recovering Wisconsin and Michigan populations. Genetic analysis of extant *Canis* samples from the western GLR, based on a variety of genetic markers, supports *lupus/lycaon* hybridization (see Chapter 2), as does previous research (Mech and Federoff 2002; Wilson *et al.* 2008). The hypothesis that the current Great Lakes wolf population is derived from *lupus/latrans* hybridization is rejected by the data (Figure 3.2).

The conclusion that the recovered Wisconsin and Michigan wolf populations are composed of *C. lupus-lycaon* hybrids has implications for the de-listing of gray wolves in the western GLR, and an important issue to consider is when the *lupus/lycaon* hybridization occurred. Given the genetic similarities between the pre-recovery and extant western GLR wolves, current and future conservation and management actions should focus on conserving the current wolf population and maintaining gene flow across its range, and not attempt to interfere with hybridization dynamics in the hope of achieving a "pure" animal or desired phenotype.

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FIGURE 3.2

Chapter 4: General Discussion

Summary of Findings

Genetic analyses conducted on *Canis* samples from the western Great Lakes Region (WGLR) and surrounding localities indicate that the extant wolves occupying northwestern Ontario, Minnesota, and the recovered populations of Wisconsin and Michigan represent canids derived from hybridization of gray wolves (*C. lupus*) and eastern wolves (*C. lycaon*). The genetic data indicate that wolves do not hybridize with coyotes (*C. latrans*) in the WGLR, unlike in southeastern Ontario where *latrans/lycaon* hybridization has been extensive (e.g. Wilson *et al.* 2008), congruent with morphological studies (see Nowak 1995). Genetic analysis of three century-old wolf samples from the pre-recovery WGLR population revealed that they had similar DNA profiles to wolves that currently occupy the region. This finding was interpreted to suggest that hybridization occurred between eastern wolves and gray wolves over a century ago. The extant animals in the WGLR were determined to be representative of animals that occupied the region historically, suggesting that wolf recovery efforts have been successful in the WGLR.

Discussion of Canis in Northeastern North America

The gray wolf historically occupied the majority of North America with the exception of the eastern deciduous forests, which were occupied by the eastern wolf (Nowak 1995 & 2002). The *C. lupus-lycaon* hybrids that occupy the current WGLR wolf population likely resulted from historic hybridization between eastern wolves and gray wolves. The WGLR represents a region where their distributions likely overlapped

(Nowak 2002). This hybridization may have occurred during interglacial periods of the Pleistocene when they came into contact after prior isolation. Alternatively this hybridization may be more recent, having occurred following European colonization, which may have led to the breakdown of post-glacial reproductive barriers previously maintained by habitat and prey specificities (Kolenosky and Standfield 1975; Nowak 2002). The occurrence of *lupus/lycaon* hybridization over a century ago seems plausible given that 1) gray wolves decreased in abundance in the western Great Lakes states due to human persecution since the mid 1800s (FWS 2007) and 2) eastern wolves expanded their range into this region following the northward expansion of white-tailed deer (Kolenosky and Standfield 1975; Nowak 2002). These two points suggest that the two species probably came into contact in disproportionate abundances or at low density and under disrupted population and social structure, and these factors may have contributed to hybridization. However further investigation is required to determine if this hybridization occurred pre- versus post-European colonization, and this assessment may be accomplished by the analysis of linked microsatellite loci, which can provide an estimate of the time since admixture between populations (Falush *et al.* 2003). The conservation merit of a natural hybrid population is likely perceived as greater than that of a hybrid population resulting from human influences (e.g. Allendorf *et al.* 2001). However, the cause of hybridization should not preclude sustaining a hybrid wolf population that fills the ecological role of a top canid predator in the WGLR, especially considering that unhybridized forms of the parental species likely no longer exist in the hybrid zone that encompasses the region. Similar logic applies to the conservation merit of eastern coyotes.

The eastern wolf is the mediator of *Canis* hybridization in northeastern North America, breeding with both gray wolves and coyotes in the northwest and southeast portions of its range respectively (see Chapter 2; Kyle *et al.* 2006). The ability of eastern wolves to hybridize with gray wolves, an ability coyotes apparently lack, is likely facilitated by a combination of ecological and biological factors, including similar social structure and prey selection of both species (i.e. they are both wolves), and a relatively closer evolutionary relationship than between gray wolves and coyotes (e.g. Wilson *et al.* 2000). Eastern wolf genetic material occurs in eastern coyotes ranging from the northeastern United States (Way *et al.* submitted) into southeastern Ontario and southern Quebec (Grewal 2001), and eastern wolf genetic material occurs in *C. lupus-lycaon* hybrids ranging from Quebec across northern Ontario and the western Great Lakes states into Manitoba (see Chapter 2; Grewal 2001). The last putatively pure population representative of the eastern wolf is thought to reside in Algonquin Park, however, introgression of both coyote and gray wolf genetic material into the park animals (Grewal *et al.* 2004) suggests that the eastern wolf may not exist anywhere today in its original unhybridized form. Rather, the eastern wolf genome encompasses a broad geographic range, occurring in varying proportions in *C. lupus-lycaon* hybrids and eastern coyotes. However, the Algonquin Park ecosystem appears to be selecting for wolf-like animals and against coyote-like animals, showing limited gene flow to animals south of the park (Grewal *et al.* 2004), although it may be that eastern wolves first occupied and saturated the park and have naturally excluded coyotes, which would not necessarily represent selection. Regardless, the wolves of Algonquin Park are connected by gene flow to the larger wolf population that extends to the northwest and northeast of the park (Grewal *et*

al. 2004), suggesting they do not represent a unique island population. Notwithstanding the previous statement, it could be argued that Algonquin Park wolves do exhibit notable morphological and genetic distinctiveness to canids from surrounding areas (see Chapter 2; Sears *et al.* 2003). Although a distinct species status cannot be justified for the park animals, their quasi-distinctiveness may reflect adaptation to the relatively stable park ecosystem, which may have maintained, or currently selects for, traits approximating those of the original eastern wolf in its unhybridized form. In general, one can only speculate, because unfortunately it is likely that no adequate sample of pure, unhybridized eastern wolves exists for comparison to the current park animals.

The possibility of a three-way hybrid complex may be a potential concern in northeastern North America, however, the nuclear DNA data from this study and others (see Kyle *et al.* 2006) indicate that gray wolves and coyotes do not interbreed. More specifically, *C. lupus-lycaon* hybrids do not interbreed with *C. latrans* or *C. latrans-lycaon* hybrids (i.e. eastern coyotes), or at least have not done so extensively in the region studied. However, it is recognized that the ranges of *C. lupus-lycaon* hybrids and eastern coyotes converge around central Ontario and in Quebec, and the potential for interbreeding between these two types of hybrids exists in these regions.

A large wolf population exists that extends across Quebec, Ontario, the western Great Lakes states, and Manitoba. This population represents a cline of sizes and phenotypes resulting from hybridization of *C. lycaon* with both *C. lupus* and *C. latrans* (see Kyle *et al.* 2006), and considering the high level of gene flow it is likely that some of the variation reflects ecological adaptation. The population contains genetic material of all three *Canis* species, and as a result maintains a high degree of genetic variation and

adaptive evolutionary potential. Changes in landscape conditions and the distribution and abundance of prey are factors that will likely lead to selection for more wolf-like or more coyote-like animals (e.g. Geffen *et al.* 2004; Pilot *et al.* 2006).

Conclusions

The predicted changes in climate, due to global warming, will no doubt have a significant effect on the habitat and prey distribution of canids in North America. Ensuring that wolves are saturated in the landscape may be critical to limiting potential hybridization with eastern coyotes, the range of which could expand further north into regions currently dominated by wolves. However, if landscape conditions favour the selection of wolf-coyote hybrids it can be argued that such hybridization should not be impeded, to allow the animals to effectively adapt to their environment (e.g. Kyle *et al.* 2006). These issues will no doubt be important in determining the future distribution and dynamics of *Canis* species in North America. Current conservation and management plans for wolf populations in northeastern North America should focus on conserving habitats favourable to wolves in general (i.e. large wolf-like canids), and their prey species, and conserving genetic connectivity among wolf populations to maintain adaptive evolutionary potential and ensure their long-term persistence.

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