Genetic Characterization of Hybrid Wolves across Ontario

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Abstract

Four "races" of wolves have been described in Ontario as follows: 1) *Canis lupus hudsonicus* inhabiting the subarctic tundra, 2) A race (Ontario type) of the eastern timber wolf (*Canis lupus lycaon*) that inhabits the boreal forests, 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) in central Ontario that has been proposed to be a hybrid between the Algonquin type wolf and expanding coyotes, *Canis latrans*. Using mitochondrial DNA (mtDNA) control region sequences and 8 microsatellite loci, we developed DNA profiles for 269 wolves from across Ontario. The distribution of mtDNA was predominantly coyote and the eastern wolf, *Canis lycaon*, in Algonquin Park and the southern Frontenac Axis with a combination of these mtDNA and gray wolf mtDNA in northern Ontario. Bayesian clustering grouped northern Ontario wolves independent of mtDNA with a second grouping of eastern and Tweed wolves from Algonquin. Individual clustering identified 3 groups represented by 1) northern Ontario wolves, 2) eastern wolves, and 3) Tweed wolves from the Frontenac Axis. Genomic representation analyses indicate that the Tweed wolves are hybrids between the coyote and the eastern wolf and represent the Ontario distribution of the eastern coyote, whereas the wolves in the upper Great Lakes region represent products of historic and/or continuing hybridization between *C. lycaon* and *C. lupus*. There was low structuring among wolves in these regions, and Algonquin suggesting a larger northern connected metapopulation with gene flow between the Ontario and Algonquin types.

Key words: *Canis lycaon, eastern wolf, genetics, gray wolf, hybridization*

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 western 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 gray wolves. In 1975, Kolenosky and Standfield recognized 4 types or "races" of wolves in Ontario (Figure 1A) based on skull morphology. In the subarctic tundra along the coasts of James and Hudson Bay, the subspecies of gray wolf, *Canis lupus hudsonicus*, was identified. In the boreal forest of the Hudson Bay lowlands, they recognized a race of another subspecies of gray wolf, *Canis lupus 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 "Tweed wolf" was proposed 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, *Canis lupus*, is thought to have originated in the Old World (OW) and migrated to the New World (NW) via the Bering Land bridge during the Illinoian period of the Pleistocene glaciation, some 300 000 years ago (Nowak 1979; Kurten and 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, based on the above phenotypic characters, that included *Canis lycaon* (eastern Canada) and *Canis floridanus*, *Canis lupus* var. *rufus*, and *Canis frustror* that were later recognized as subspecies of the red wolf, *Canis rufus*. Following this assessment by Miller, taxonomic surveys examine skull morphology. Pockock (1935) recognized many of the species of Miller (1912) as subspecies of the gray wolf, *C. lupus*, but maintained the eastern timber wolf as *C. lycaon*. Following a number of revisions, Goldman (1944) produced a comprehensive
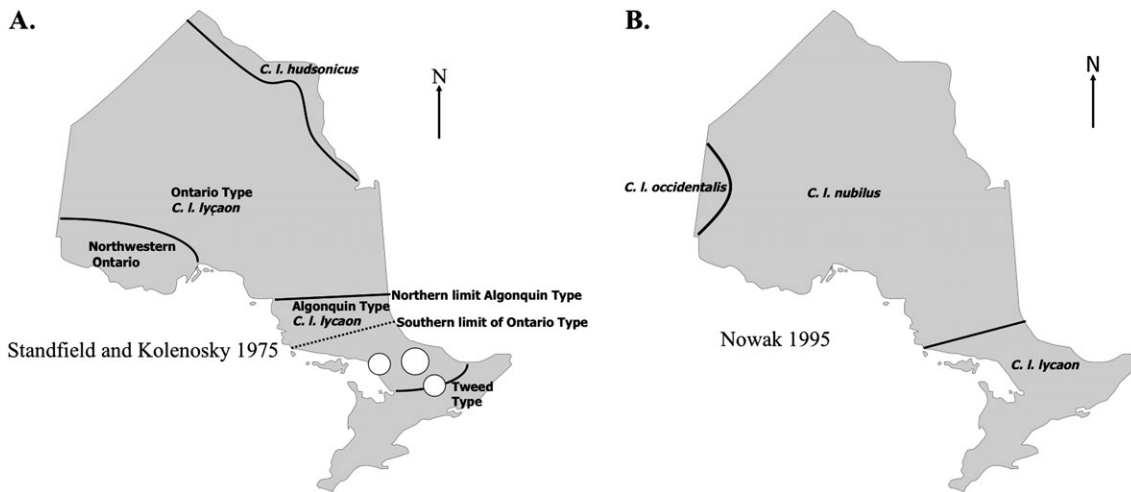
Figure 1. Previous assessments of the distribution of wolf types in Ontario. (**A**) Kolenosky and Standfield (1975) showing the estimated northern limit of the Algonquin type and southern limit of the Ontario type in central Ontario. The larger circle represents Algonquin Provincial Park, and the smaller circles are locations of Tweed wolves. (**B**) *Canis lupus* subspecies distribution proposed by Nowak (1995) showing most of Ontario occupied by the gray wolf subspecies *Canis lupus nubilus* that he also placed throughout the Plains States of the United States as well as the more limited distribution of the original designated *Canis lupus lycaon* within central Ontario.

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, however, Nowak (1983) and Mulders (1997) concluded that *C. l. hudsonicus* should be reversed to *Canis lupus occidentalis*. Nowak (1983, 1995) further suggested grouping North American wolves into 5 subspecies based on Pleistocene refugia, with 3 of these occurring in Ontario based on similarities of skull measurements, *C. l. occidentalis*, *Canis lupus nubilus*, and *C. l. lycaon* (Nowak 1995; Figure 1B). Based on this proposed distribution, most of Ontario was inhabited by *C. l. nubilus*, a subspecies originally assigned to the central Plains of the United States.

Earlier genetic studies (Lehman et al. 1991; Wayne and Lehman 1992; Wayne et al. 1992) of wolves in the Great Lakes region concluded that there are "hybridizing" wolf populations in northwest Ontario, Minnesota (MN), 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 timber wolf in Algonquin Provincial Park is either the same or very similar to the red wolf, *C. rufus*, and that both diverged from the coyote 300 000 years ago, whereas the gray wolf diverged more than 1 million years ago (Ma; 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; Forbes and Boyd 1997; Pilgrim et al. 1998). Additional genetic studies support the existence of an eastern North American wolf species, independent of the gray wolf (Bertorelle and Excoffier 1998; Hedrick et al. 2002; Wilson et al. 2003).

Despite the changing classification of Ontario canids, there has been little empirical consideration of the role of hybridization and the potential barriers to gene flow that originally must have been present to cause and maintain the differences among the types (Kolenosky and Standfield 1975) or subspecies (Nowak 1995). Since the arrival of the Europeans, human impacts such as deforestation, farming, trapping, poisoning, and bounty hunting extirpated wolves throughout most of Mexico, the 48 contiguous United States, and southern Canada providing opportunities for the expansion of the coyote and the subsequent breakdown of the reproductive barriers between coyotes and eastern wolves. If the eastern wolf, *C. lycaon*, represents a North American-evolved wolf (Nowak 1983; Wilson et al. 2000), a level of reproductive isolation between them and the Eurasian-evolved gray wolf, *C. lupus*, would be expected. Kolenosky and Standfield (1975) described an absence of a cline between the Ontario and Algonquin type and indicates that recognition of a barrier to gene flow, consistent with a boundary between the gray wolf and the eastern wolf, was prevalent until the 1960s. There is some evidence from descriptions of the natural history of the Adirondacks from the mid-19th century for the presence of the Algonquin (common) and Ontario (rare) types described by Kolenosky and Standfield (1975; DeKay 1842). In this study, we analyzed the control region of the mtDNA and

Figure 2. Map showing location of wolf samples from across Ontario. The samples (Table 1) were grouped into 6 regions in order to examine the types described by Kolenosky and Standfield 1975. Northwest Ontario, northeast Ontario, Pukaskwa National Park, Algonquin Provincial Park, Magnetawan region to the west and north of Algonquin Provincial Park, and the Frontenac Axis to the west and south of Algonquin Provincial Park.

8 microsatellite loci in Ontario wolf samples. The primary objective of the study was to understand the genetic relationships of the 4 wolf types identified by Kolenosky and Standfield (1975) in the context of the coyote (*C. latrans*) and 2 distinct wolf species, the gray wolf (*C. lupus*) and the eastern timber wolf (*C. lycaon*).

Materials and Methods

Sample Collection and DNA Extraction

We analyzed 269 samples from 6 geographic regions within Ontario (Figure 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 ($n = 30$); and Pukaskwa National Park ($n = 13$; Table 1). For the purpose of this study, the designations of northeastern and northwestern Ontario describe the boreal distribution in the province (Figure 2). DNA from blood and tissue samples was extracted by standard phenol–chloroform extraction methods described in Guglich et al. (1994). Additional samples were included in specific analyses in this study: gray wolves from Northwest Territories (NWT; $n = 66$); wolves from MN ($n = 9$); coyotes from

Saskatchewan (SK; $n = 36$), North Carolina (NC; $n = 22$), Texas (TX; $n = 26$), and Ohio (OH; $n = 22$); and eastern coyotes from the Adirondacks ($n = 66$) and a neighboring region in New York (NY; $n = 24$), Maine (ME; $n = 102$), and New Brunswick (NB; $n = 20$). These additional specimens were included as representative of nonhybridizing *Canis* (i.e., gray wolves from NWT and coyotes from SK, TX, NC, and OH) and those from reported areas of hybridization (i.e., MN Great Lakes wolves and eastern coyotes from NY, NB, and ME) outside of Ontario (for sampling information, see Supplementary Table 1).

mtDNA Analysis

Identification of NW (C. lycaon/C. latrans) and OW (C. lupus) mtDNA

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- to 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 10 μl per tube using 25 ng of genomic DNA, 200 μM dNTPs, 1× amplification buffer, 2 mM MgCl$_2$, primers 1 and 2 (0.2 μM), and 0.5 units

Table 1. Sample information including geographic location, number of samples, type of biological material, and the source of the submitted material

Area[a]	Number	Type	Source
Frontenac Axis	74	Muscle	University of Waterloo[b]
Algonquin Provincial Park (1960s)	19	Teeth	Ontario Ministry of Natural Resources
Algonquin Provincial Park (1990s)	92	Muscle and blood	University of Waterloo[b]
Magnetawan region	26	Muscle	University of Waterloo[b]
Northeastern Ontario (1960s)	46	Teeth	Ontario Ministry of Natural Resources
Northeastern Ontario (1990s)	34	Hide	North Bay Fur House
Pukaskwa National Park	13	Blood	Parks Canada[c]
Northwestern Ontario (1960s)	11	Teeth	Ontario Ministry of Natural Resources
Northwestern Ontario (1990s)	30	Hide	Laurentian University[d]
MN	9	Muscle	University of Minnesota[e]
Total	354		

[a] Location of area shown in Figure 2.

[b] University of Waterloo samples—provided by Dr. J. Theberge.

[c] Provided by Dr P. Paquet and F. Burrows.

[d] Laurentian University samples provided by Dr F. Mallory.

[e] University of Minnesota samples provided by Dr L. D. Mech.

of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94 °C for 5 min, 55 °C for 30 s, and 72 °C for 30 s (1 cycle); 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s (35 cycles); and 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min (1 cycle). Products were then mixed with 0.4 volume of formamide loading buffer and were heated at 95 °C for 5 min 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.

Genetic Characterization of Microsatellite Loci

We analyzed allele frequencies at 8 microsatellite loci (Ostrander et al. 1993; Roy et al. 1994, 1996) as per Wilson et al. (2000) for 225 individual canids representative of the different geographic regions of Ontario. Within the 225, a total of 49 unrelated eastern timber wolves were used in the microsatellite analyses to represent Algonquin Provincial Park animals. Microsatellite loci genotypes were assigned based on the allele sizes of Roy et al. (1994, 1996). Allelic diversity (A), the effective number of alleles (A_E), and expected heterozygosity (H_E) were estimated using the software program GENALEX 6 (Peakall and Smouse 2006; Table 1). F_{IS} (Weir and Cockerham 1984) were generated using the program GENETIX (Belkhir et al. 1988). Significant deviation from Hardy–Weinberg Equilibrium for each population–locus combination was tested for each region using the Markov Chain method in GENEPOP 3.1 (Guo and Thompson 1992; Raymond and Rousset 1995) and applying a sequential Bonferroni correction for multiple statistical tests (Rice 1989).

Bayesian Cluster Analysis

Population structure was assessed with 2 Bayesian methods: first, the Bayesian analysis of population structure (BAPS 2.0; Corander et al. 2003, 2004) and the model-based clustering method STRUCTURE (Pritchard et al. 2000; Falush et al. 2003).

The BAPS method (Corander et al. 2003, 2004) was used to cluster sampling regions into larger population groups. Prior information included the region where a sample was collected and in areas with overlapping mtDNA types (i.e., OW evolved or gray wolf and NW evolved or eastern wolf and coyote), samples were grouped according to this classification. Populations were partitioned following 10^5 iterations following a burn-in period of 10^4. The maximum posterior probability (p(S|data) more than the data set was estimated to identify the single most significant partition.

Where BAPS clusters sampled populations, STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) clusters individuals and does not require an "a priori" assignment of individuals based on geography or taxonomy and identifies genetically similar multilocus genotypes for individuals without any known population or taxonomic affiliation and provides a statistical assessment of the number of genetic clusters or subpopulations (K). Individuals are then assigned probabilistically to clusters, and individuals with admixed ancestry are assigned to more than 1 group (K). STRUCTURE has been previously applied to identifying hybrids in canids (Randi and Lucchini 2002; Lucchini et al. 2004) and other taxa (Randi et al. 2001) and the genetic structure and admixture of domestic dogs (Parker et al. 2004).

We generated ancestry coefficients (q_i; probability of assignment or membership to a specific cluster) of individuals by estimating the posterior probabilities assuming prior values on K between 1 and 10 (option MAXPOPS = 1–10) with triplicate runs. Bayesian models were modeled with uninformative priors on all the iterations of K with pooled data sets (option USERPOPINFO = 0) and allowing for asymmetric admixture by estimating the level of admixture (alpha) for each population (POPALPHA = 1). We applied 10^6 iterations with a 10^5 burn-in period in

Table 2. Distribution of NW and OW mtDNA control region in Ontario across the 6 geographic regions in Ontario

Population	Frontenac Axis	Magnetawan region	Algonquin Provincial Park		Northeastern Ontario		Northwestern Ontario		Pukaskwa National Park
	1990	1990	1960	1990	1960	1990	1960	1990	1990
OW	0	0	0	4	22	18	1	9	11
NW	74	26	19	88	24	15	10	21	2
Total	74	26	19	92	46	33	11	30	13

The total number coincides with the number of individuals used in this study.

triplicate assuming an F model of admixture and correlated allele frequencies to determine individual ancestry and the likelihood of the number K within the data set (Pritchard et al. 2000; Falush et al. 2003; Evanno et al. 2005). Graphical displays of individual coefficients of membership (ancestry) were generated using the software program DISTRUCT (Rosenberg et al. 2001).

Although Bayesian analysis provided an assessment of clustering at the population and individual levels, we estimated indirect measures of population structure to quantify the relationship among regions in Ontario. Gene flow was estimated among sampled regions using F_{ST} with the software program GENETIX (Belkhir et al. 1988) and R_{ST} with the software program ARLEQUIN (Schneider et al. 2000). R_{ST} is applied to loci undergoing a stepwise mutation process permitting homoplasy where 2 alleles of the same size can occur independently in 2 populations. This is most appropriate for this data set as 2 lineages are being assessed, *C. lupus* (OW) and *C. lycaon/C. latrans* (NW), having diverged 1–2 Ma.

Results

Identification of NW (C. lycaon/C. latrans) and OW (C. lupus) mtDNA

Initially, the genetic characteristics of Ontario wolves were assessed based on the geographic regions from which they were sampled (Table 1). The animals in the Frontenac Axis and Magnetawan regions contained only NW mitochondria corresponding to either *C. latrans* or *C. lycaon* (Table 2). Hybridization between western coyotes and eastern wolves

Table 3. Geographic region, sample sizes (N), average number of alleles ($A \pm$ standard error [SE]), and expected heterozygosity ($H_E \pm$ SE) estimated with the software program GENALEX (Peakall and Smouse 2006) and F_{IS} (Weir and Cockerham 1984) estimated using the software Genetix (Belkhir et al. 1988)

Geography	N	A	H_E	F_{IS}
Algonquin	49	6.62 ± 0.78	0.644 ± 0.061	0.001
Frontenac	74	7.63 ± 1.03	0.707 ± 0.056	0.039
Magnetawan	26	6.50 ± 0.57	0.688 ± 0.049	0.036
Northeastern Ontario	34	7.12 ± 0.95	0.724 ± 0.043	0.020
Pukaskwa	13	5.00 ± 0.50	0.673 ± 0.029	−0.003
Northwestern Ontario	30	6.87 ± 0.88	0.712 ± 0.033	0.028
MN	9	4.38 ± 0.42	0.658 ± 0.032	−0.068

does not allow the diagnostic assignment of the NW-evolved species based on mtDNA haplotype. Only 4 of 92 animals from Algonquin Provincial Park contained OW mitochondria or *C. lupus* haplotypes consistent with a predominantly eastern wolf (*C. lycaon*) ancestry. Most of the animals in Pukaskwa National Park contained OW mitochondria consistent with a predominant gray wolf ancestry (*C. lupus*). There was a mixture of animals with OW and NW mitochondria from northwestern and northeastern Ontario.

Genetic Characterization of Microsatellite Loci

Allelic diversity, heterozygosity, and F_{IS} values were calculated for 8 microsatellite loci from each of the regions, and these estimates of genetic variation values were similar among the various populations (Table 3). No loci were significantly different from Hardy–Weinberg expectations following correction for multiple population–locus combinations.

Bayesian Cluster Analysis

BAPS compared regional groupings of North American wolves and coyotes and identified 2 clusters associated with Ontario: a northern cluster that grouped with MN wolves and eastern wolves from Algonquin Park with canids from Magnetawan and Tweed wolves from the Frontenac Axis (Table 4). The clustering of northern wolves into a single population was irrespective of the mtDNA classification (OW vs. NW) indicating gene flow between the different maternal lineages.

Applying the Bayesian STRUCTURE analysis to Ontario canids, including MN wolves, revealed 3 genetic clusters ($K =$ 3) based on the estimated Ln likelihood (Pritchard et al. 2000; Falush et al. 2003) and second order rate of change of the likelihoods (Evanno et al. 2005). The inferred genetic clusters corresponded to the northern wolf group identified with BAPS; an Algonquin eastern wolf cluster; and a Tweed wolf cluster. In addition to increasing the resolution of identifying the number of genetic clusters, STRUCTURE revealed ancestry assignments at the individual level (Pritchard et al. 2000; Falush et al. 2003). The discordance between BAPS and STRUCTURE in the number of inferred clusters for Ontario specimens only, $K = 2$ versus $K = 3$, respectively, likely resulted from the geographic overlap of individuals from different clusters that generated allele frequency similarities resulting in a single grouping with BAPS. This applied to the inferred eastern wolf and Tweed wolf clusters (Figure 2). Although Algonquin Park and the Frontenac Axis were inhabited by their predominant wolf type (eastern wolves and Tweed wolves, respectively),

Table 4. The partition of wolf and coyote populations with maximum posterior probability (p(S|data = 0.9927)

Cluster 1	NWT
	MN, northwestern Ontario (OW mtDNA), northwestern Ontario (NW mtDNA), Pukaskwa National Park, northeastern Ontario (OW mtDNA), and northeastern Ontario (NW mtDNA)
Cluster 2	Algonquin Provincial Park, Frontenac Axis, and Magnetawan in Ontario
Cluster 3	Adirondacks and Cortlandville in NY, NB, and
Cluster 4	ME
Cluster 5	SK, NC, TX, and OH

some animals from the neighboring cluster were observed in these geographies. This overlap extended into the Magnetawan region which also contained individuals and admixture from the northern wolf cluster (Figure 2).

A second area of increased admixture of high-ancestry individuals from different clusters was observed in the northeastern Ontario animals with NW mtDNA (Figure 3). Although the majority of wolves originated from the northern cluster, some individuals were of eastern wolf and Tweed wolf ancestry. This was in contrast to the animals with OW mtDNA that predominantly clustered with the northern boreal wolf group that extended into Pukaskwa, northwestern Ontario, and MN where the ancestry of the majority of individual wolves was independent of their mtDNA classification (Figure 3).

Mapping the geographic structure of ancestries over the landscape (Figure 4) revealed a general south-to-north cline trend of a Tweed wolf, an Algonquin Park eastern wolf, and an upper Great Lakes wolf. This stratification is concordant with the types or races described by Kolenosky and Standfield (1975): the Tweed wolves, the Algonquin wolf type, and the Ontario type wolf. Based on the introgression of genetic material inferred from individual ancestries and the distribution of mtDNA, these wolf types correspond to taxonomic hybrid designations of *C. latrans* × *C. lycaon* for the Tweed wolf, *C. lycaon* × *C. latrans* for the eastern wolves in Algonquin Park, and *C. lupus* × *C. lycaon* or vice versa in

the northern regions of Ontario. The order of the species designation denotes the relatively higher and lower genome proportion of the parental species.

To obtain quantitative estimates of connectivity and gene flow among geographic regions, we assessed population structuring using R_{ST} (Slatkin 1995) and F_{ST} (Weir and Cockerham 1984; Table 5). R_{ST} provides better assessments of differentiation among divergent populations or taxa, whereas F_{ST} is more representative at less differentiated intraspecific comparisons. The majority of the comparisons in this study range are between *Canis* species and their related hybrids, and as a result, R_{ST} is expected to be a more representative indirect estimate of population structure.

The R_{ST} value between Tweed wolves in the Frontenac Axis and eastern wolves from Algonquin Park was relatively high (Table 5). 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. Surprisingly, the animals from northeastern Ontario, in the area east of Lake Superior, showed marked structuring with the wolves found in the adjacent Pukaskwa National Park and much less with the more distant Algonquin Park eastern wolf population. Even more surprising was the lack of structuring of the northwestern Ontario animals and the animals in Algonquin Provincial Park.

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 presumably also their gray wolf (*C. lupus*) predators and allowed the northern advancement of deer (*Odocoileus virginianus*) 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
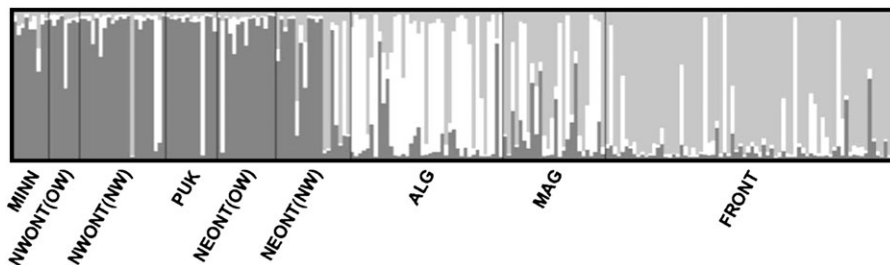
Figure 3. Bayesian clustering assignment of the estimated proportion of membership (i.e., ancestry) of Ontario wolves from Minnesota (MINN), northwestern Ontario (NWONT), Pukaskwa National Park (PUK), northwestern Ontario (NEONT), Algonquin Provincial Park (ALG), the Magnetawan region (MAG), and the Frontenac Axis (FRONT) for $K = 3$. Individuals with OW *Canis lupus* mtDNA or NW *Canis latrans/lycaon* mtDNA are identified. Each individual is represented by a vertical line partitioned into colored segments. Dark gray corresponds to gray wolf ancestry, light gray to coyote ancestry, and white to eastern wolf ancestry.
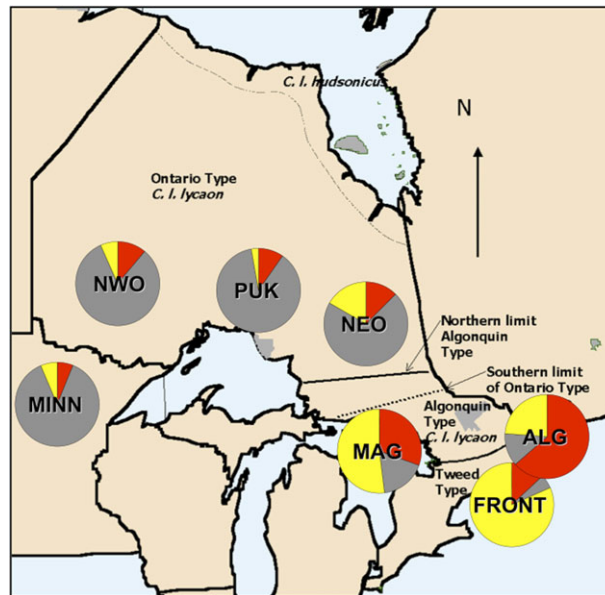
Figure 4. Proportions of ancestry of each geographic region defined with the model-based method STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). Gray corresponds to gray wolf ancestry, red to eastern wolf, and yellow to coyote ancestry.

1970a, 1970b) and allowed the spread of the coyote throughout the newly created farmland and the subsequent hybridization with *C. lycaon*.

The genetic data are consistent with the hypothesis that the Tweed wolf as described by Kolenosky and Standfield (1975) and Schmitz and Kolenosky (1985) is a hybrid between the Algonquin type wolf and the coyote. However, in contrast to Kolenosky and Standfield, recent genetic evidence (Wilson et al. 2000) suggests that these hybrids originated from interbreeding between 2 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 event explains the anomaly of the lack of interbreeding between western coyotes and gray wolves (Roy et al. 1994; Wilson et al. 2000). As Schmitz and Kolenosky (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 represents the Ontario distribution of the eastern coyote (Moore and Parker 1992) that is extremely adaptable

to both agricultural and low-density forested habitats. Despite the high numbers of the Tweed wolves, southeast of Algonquin Park, the R_{ST} data (Table 5) support the proposition that barriers to gene flow exist by maintaining larger wolflike animals within the Park. Despite relatively less gene flow from the Frontenac Axis, the high level of genetic variation in Algonquin Park (Table 2) is supported by the gene flow from the Magnetawan region, northeastern Ontario, and Quebec (Grewal et al. 2004). Although the Algonquin Park population numbers less than 200, evidence suggests that it is part of a larger metapopulation that includes animals from northeastern and northwestern Ontario and Quebec (Grewal et al. 2004; Kyle et al. 2006).

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. The low R_{ST} values between the northeastern and northwestern animals indicate that there is substantial gene flow between both regions of the province. However,

Table 5. Estimates of F_{ST} (lower diagonal) (Weir and Cockerham 1984) and R_{ST} (upper diagonal) (Slatkin 1995) for pairwise comparisons among geographic regions in Ontario

	ALG	MAG	FRONT	NEONT	PUK	NWONT	MINN
ALG	0.000	0.047	0.113	0.018	0.254	0.002	0.008
MAG	0.022	0.000	0.048	0.029	0.278	0.047	0.074
FRONT	0.055	0.024	0.000	0.129	0.365	0.130	0.099
NEONT	0.073	0.053	0.078	0.000	0.171	0.029	0.048
PUK	0.117	0.088	0.118	0.057	0.000	0.198	0.290
NWONT	0.071	0.057	0.094	0.014	0.055	0.000	0.007
MINN	0.089	0.069	0.105	0.036	0.106	0.022	0.000

it is difficult to make any distinction between the relative species' contribution to these wolf hybrids in the upper Great Lakes regions, specifically whether the northern Ontario wolves represent *C. lycaon* × *lupus* or *C. lupus* × *lycaon* or which wolf has the higher proportion of genome ancestry. This is particularly difficult given the hybridization between eastern wolves and expanding western coyotes in the formation of the Tweed wolf and historic introgression of western coyote genetic material into Algonquin eastern wolves. Despite this limitation, it is clear that *C. lycaon* genetic material exists in multiple wolf types with varying levels of gene flow throughout Ontario. As a result, this complex interspecific metapopulation is large, numbering in the thousands rather than the hundreds.

In northwestern Ontario, populations appear to be genetically related to animals in Algonquin Provincial Park; however, there appears to have been less hybridization with the Tweed eastern coyote based on ancestries (Figures 3 and 4). Furthermore, the high ancestries of the Ontario type wolves in northwestern Ontario and MN regions regardless of mtDNA suggests past introgressive hybridization and less recent admixture between *C. lupus* and *C. lycaon* (Figures 3 and 4). A divergent *C. lycaon*-specific mtDNA haplotype in neighboring Manitoba (Wilson et al. 2000, 2003) supports divergence of *C. lycaon* in the upper Great Lakes region from its southern counterparts. This pattern may reflect a longer history of hybridization between eastern wolves (*C. lycaon*) and gray wolves (*C. lupus*).

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). We propose that this cline is likely related to the introgression of more coyote genetic material in the south and eastern wolf × gray wolf hybridization in northern Ontario. The introgression of genes may further be influenced by selection based on factors such as prey size (Hillis 1990; Mulders 1997). *Canis lycaon* within Algonquin Park prey predominantly on white-tailed deer and beaver (*Castor canadensis*; Forbes and 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 likely reflect the "purest" gray wolf form (*C. lupus*) currently sampled based on the proportion of OW mtDNA and the relatively higher population structuring between the gray wolves in the park and the adjacent hybrid wolves. Pukaskwa wolves prey primarily on moose and appear to be surrounded by the larger Ontario type animals in patchy habitat that contains moose and white-tailed deer.

Although, the genetic data show congruence with the Algonquin and Ontario races or types described by Kolenosky and Standfield (1975), it is currently difficult to infer the presence and extent of northern *C. l. hudsonicus*

subspecies. It is reasonable to infer that this wolf is of gray wolf (*C. lupus*) origin; unfortunately, we had few samples from the far north of Ontario to assess whether the Pukaskwa National Park animals were representatives of gray wolves of the Hudson Bay Lowlands. Increased northern Ontario representation is recommended to resolve the delineation of gray wolf × eastern wolf hybridization as well as estimating the relative contribution of wolf species to the hybrid complex in the province.

In summary, the genetic data are consistent with the hypothesis that the Tweed wolf is a hybrid between the coyote and eastern wolf (*C. latrans* and *C. lycaon*). The eastern wolf represents the Algonquin type described by Kolenosky and Standfield (1975). 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. 2004), and the total number of animals may be in the thousands. Pukaskwa National Park contains a small semi-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 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 3 species.

The findings of this study support the presence of an extensive hybrid zone between eastern wolves and western coyotes (*C. latrans*) and eastern wolves and gray wolves (*C. lupus*). Lack of hybridization between western gray wolves and western coyotes in the absence of eastern wolves (Roy et al. 1994; Forbes and Boyd 1997; Pilgrim et al. 1998) coupled with recent genetic findings (Bertorelle and Excoffier 1998; Hedrick et al. 2002; Wilson et al. 2003) supports the interpretation of an eastern North American wolf species that is more closely related to the coyote and that evolved independently of the gray wolf. The relationship among eastern wolves, coyotes, and gray wolves has important implications on a number of conservation-related issues such as the status of Ontario gray wolves, the reintroduction of wolves into the northeastern United States, and the red wolf reintroduction program.

First, the current distribution of nonhybridized gray wolves in Ontario is best represented by Pukaskwa National Park, although initial hybridization is evident within the park. We suggest that the larger eastern wolf metapopulation has expanded northward with increasing hybridization with gray wolves. Additional sampling and profiling is attempting to map the distribution of the eastern wolves and gray wolves in Ontario to find the northern limit of the eastern wolf. Second, although this study has focused on Ontario, similar interbreeding among canids is likely occurring in Manitoba, the upper

Great Lake states, and Quebec. This extended distribution of the eastern wolf has implications for Canada's Committee on the Status of Wildlife in Canada process under newly approved Species at Risk Act and the US Endangered Species Act.

Currently, the only canid included on the US endangered species list in the northeastern United States is *Canis lupus*, but there is no formal plan for reintroducing the species there. The red wolf (*C. rufus*) in the southeastern United States is listed, and a multimillion dollar restoration program has been underway there since 1987, with about 100 wolves currently free ranging in North Carolina (US Fish and Wildlife Service). Some of the red wolves have been hybridizing with eastern coyotes and attempts are underway to control coyotes in red wolf range to prevent that.

Our findings (but cf., Nowak 2002) suggest that the following changes should be considered in the formal US designations and programs: 1) the northeastern United States should be excluded from the range of *C. lupus* in the endangered species listing, and the range of the red wolf (*C. rufus* or *C. lycaon*) should be extended into that area; 2) part of the northeastern United States should be considered for any new red wolf reintroductions; and 3) eastern coyotes and red wolves in the United States should be allowed to hybridize as they are naturally doing along the Algonquin–Frontenac interface in Ontario.

In Canada, the eastern wolf is listed as special concern despite its potential extensive range, and the attention devoted to this wolf may be at the expense of the northern gray wolves in Manitoba, Ontario, and Quebec, which our data suggest may be declining as eastern wolves expand. Furthermore, in both Canada and the United States, the ability to estimate the numbers of the respective canids, required for status considerations in both countries, is difficult given the past and present levels of hybridization. This issue extends to any enforcement of the protection assigned to one or more of these *Canis* species in the ability to distinguish an animal as coyote, eastern wolf to gray wolf, or an introgressed form of these species.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Funding

Acknowledgments

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