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

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Abstract: The origin and taxonomy of the red wolf (*Canis rufus*) have been the subject of considerable debate and it has been suggested that this taxon was recently formed as a result of hybridization between the coyote and gray wolf. Like the red wolf, the eastern Canadian wolf has been characterized as a small "deer-eating" wolf that hybridizes with coyotes (*Canis latrans*). While studying the population of eastern Canadian wolves in Algonquin Provincial Park we recognized similarities to the red wolf, based on DNA profiles at 8 microsatellite loci. We examined whether this relationship was due to similar levels of introgressed coyote genetic material by comparing the microsatellite alleles with those of other North American populations of wolves and coyotes. These analyses indicated that it was not coyote genetic material which led to the close genetic affinity between red wolves and eastern Canadian wolves. We then examined the control region of the mitochondrial DNA (mtDNA) and confirmed the presence of coyote sequences in both. However, we also found sequences in both that diverged by 150 000 – 300 000 years from sequences found in coyotes. None of the red wolves or eastern Canadian wolf samples from the 1960s contained gray wolf (*Canis lupus*) mtDNA sequences. The data are not consistent with the hypothesis that the eastern Canadian wolf is a subspecies of gray wolf as it is presently designated. We suggest that both the red wolf and the eastern Canadian wolf evolved in North America sharing a common lineage with the coyote until 150 000 – 300 000 years ago. We propose that it retain its original species designation, *Canis lycaon*.

Résumé : Les origines et la taxonomie du Loup roux (*Canis rufus*) font l'objet d'une controverse importante et une hypothèse a été émise, à savoir qu'il s'agit d'un taxon récent issu de l'hybridation entre le Coyote et le Loup gris. Comme le Loup roux, le Loup de l'est du Canada est décrit comme un petit loup « mangeur de cerfs » qui s'hybride avec le Coyote (*Canis latrans*). L'étude de la population de Loups de l'est du Canada dans le parc provincial Algonquin nous a permis de reconnaître des similarités avec le Loup roux d'après les profils d'ADN à 8 locus microsatellites. Nous avons tenté de déterminer si cette relation était due à des degrés semblables d'introgression de matériel génétique en comparant les allèles microsatellites avec ceux d'autres populations nord-américaines de loups et de coyotes. Les analyses ont révélé que ce n'est pas du matériel génétique de coyote qui a mené à la grande affinité génétique entre le Loup roux et le Loup de l'est du Canada. Nous avons ensuite examiné la région de contrôle de l'ADN mitochondrial (ADNmt) et confirmé la présence de séquences du Coyote chez les deux loups. Cependant, nous avons également

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trouvé des séquences qui divergent de celles des coyotes par un écart de l'ordre de 150 000 – 300 000 années. Aucun des Loups roux ou des Loups de l'est du Canada échantillonnés au cours des années 1960 ne comptait de séquences d'ADNmt du Loup gris (*Canis lupus*). Les données n'appuient pas l'hypothèse selon laquelle le Loup de l'est du Canada est une sous-espèce du Loup gris, tel qu'on le reconnaît maintenant. Nous croyons que le Loup roux et le Loup de l'est du Canada ont évolué conjointement en Amérique du Nord, suivant une lignée commune avec le Coyote jusqu'à il y a 150 000 – 300 000 ans. Nous proposons de garder au Loup de l'est du Canada son nom scientifique actuel, *Canis lycaon*.

[Traduit par la Rédaction]

Introduction

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

The eastern Canadian wolf, *C. l. lycaon*, like the red wolf, has been the subject of several taxonomic treatments that have moved it from species status, *Canis lycaon*, to its presently accepted status as a gray wolf subspecies (Brewster and Fritts 1995). Eastern North American wolves have been described as among the smallest on the continent since the late 1700s (Goldman 1944), long before any documented arrival of coyotes (*C. latrans*) in the 1900s. As with the red wolf, there is general agreement that *C. l. lycaon* readily hybridizes with the coyote, and studies of mitochondrial DNA (mtDNA) have shown hybridization between wolf populations east of Minnesota with coyotes (Lehman et al. 1991; Wayne and Lehman 1992).

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

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

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

Materials and methods

Samples and DNA extraction

Eastern Canadian wolves, representing the putative gray wolf subspecies *C. l. lycaon*, were sampled from Algonquin Provincial Park and surrounding area from 1960 to 1965 (n = 19) and from 1985 to 1996 (n = 49). *Canis rufus* samples from the captive red wolf breeding program (n = 60) were also analyzed. Texas coyotes (n = 24) were used to represent *C. latrans*. Gray wolves, *C. lupus*, were sampled from the Northwest Territories (n = 67). DNA was extracted by methods described in Guglich et al. (1994) from frozen organ samples (liver, heart, kidney, or muscle) or from whole blood obtained by venipuncture of individuals that were livetrapped and released. DNA from the captive red wolf program, Texas coyotes, and historic teeth collected in Ontario during the 1960s was extracted following a modified Qiagen (Qiagen) extraction protocol using the lysis buffer described in Guglich et al. (1994).

Microsatellite analysis

Ten microsatellite loci (Ostrander et al. 1993; Roy et al. 1994, 1996) were amplified using 4.6 pmol γ^{33}P T4 polynucleotide kinase (Boehringer-Mannheim) end labelled primer ATP in a total reaction volume of 10 µL per tube using 25 ng of genomic DNA, 200 µM dNTPs, 1× amplification buffer, 2.0 mM $MgCl_2$, unlabelled primer (0.2 mM), 1.0 µg of bovine serum albumin (BRL), and 0.5 units of *Taq* polymerase (BRL). Products were amplified under the following conditions: 94°C for 5 min, 55–65°C for 30 s, 72°C for 15 s, 1 cycle; 94°C for 15 s, 55°C for 30 s, 72°C for 15 s, 30 cycles; 94°C for 15 s, 55°C for 30 s, 72°C for 2 min, 1 cycle. Products were then mixed with an equal volume of formamide loading buffer and 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 for the microsatellite alleles.

Control-region sequencing and sequence analysis

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

Primer 1: 5′-GAAGCTCTTGCTCCACCAATC-3′ (Pilgrim et al. 1998)

Primer 2: 5′-GGGCCCGGAGCGAGAAGAGGGAC-3′

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

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

Genetic analysis

We analyzed allele frequencies at 8 loci among the Algonquin Park and red wolf populations and compared them with the other North American populations of wolves and coyotes (Roy et al. 1994, 1996). Microsatellite alleles were assigned on the basis of size given in Roy et al. (1996). Nei's genetic distance (1972) was calculated using the programs SEQBOOT, GENDIST, and NEIGHBOR in the computer program PHYLIP (Felsenstein 1993).

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

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

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

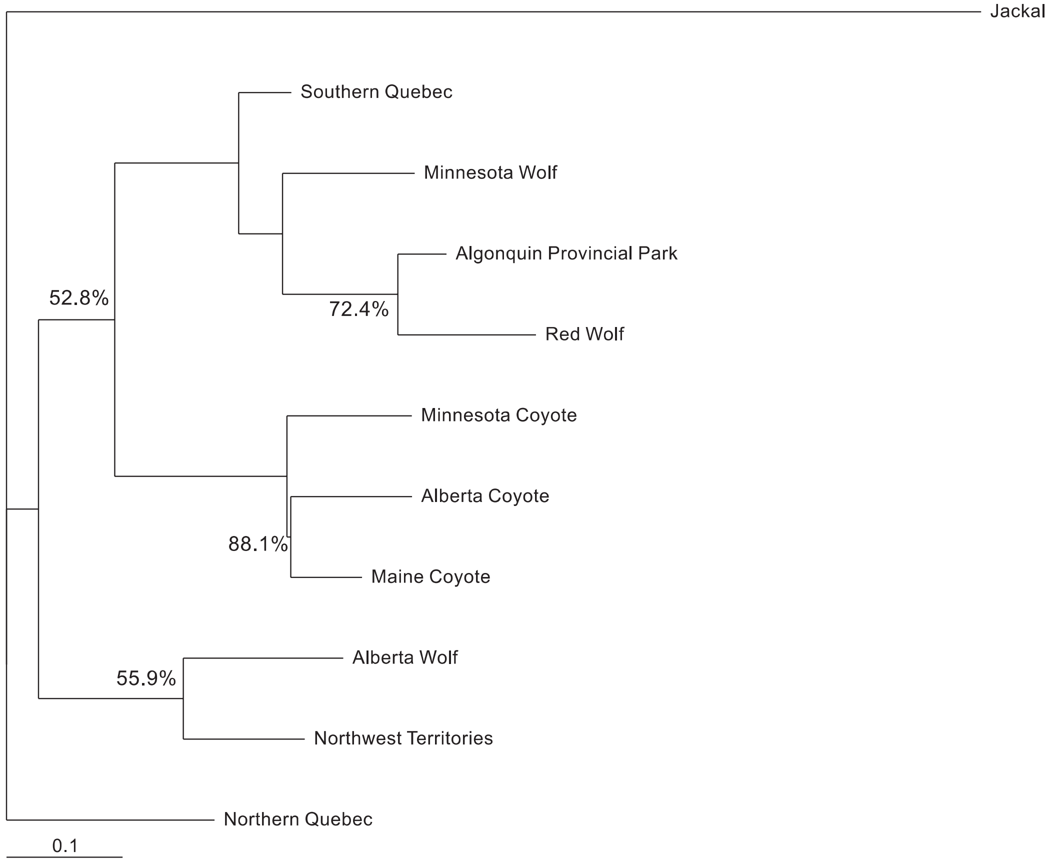
Results

The neighbor-joining analysis of genetic distances showed an unexpectedly close relationship among Algonquin Park animals, the red wolf, Minnesota wolves, and southern Quebec wolves (Fig. 1). To evaluate whether this was because "hybridizing wolves" in southern Quebec and Minnesota contain similar amounts of coyote genetic material to the red wolf (Roy et al. 1994), we determined DNA profiles of captive red wolves and other populations of gray wolves and Texas coyotes. The same relationship between eastern Canadian wolves and captive red wolves was observed when they were compared with gray wolves and Texas coyotes (Fig. 2). In this comparison, the interpretation that eastern Canadian wolves and red wolves share similar levels of coyote introgression did not seem consistent with the genetic distance between red wolves and Texas coyotes, which were the geographically closest coyote source population for the red wolf. The genetic similarity between red wolves and eastern Canadian wolves was not heavily influenced by the introgression of coyote genetic material: alleles that were prevalent in Texas and other coyote populations (Roy et al. 1994) were absent or present at very low frequency in red wolves (Table 1).

We determined the distribution of POI (Fig. 3A) and I_I values (Fig. 3B) for the captive red wolves, using allele frequencies from the Algonquin Park population, representing the eastern Canadian wolf, and from the Texas coyote population. The majority of captive red wolves overlapped the distribution of the eastern Canadian wolf population in both assignment tests. If coyote genetic material resulted in apparent similarity of these wolves, we would have expected the red wolf to fall within or closer to the distribution of its geographic neighbors, the Texas coyote population, and not that of the geographically distant population of eastern Canadian wolves in Algonquin Park.

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

Fig. 1. Neighbor-joining tree of Nei's genetic distances for allele frequencies from eight microsatellite loci. With the exception of the Algonquin Provincial Park population, the allele frequencies are from Roy et al. (1994, 1996). Two of 10 dinucleotide microsatellite loci, cxx 344 and cxx 213, from Roy et al. (1994, 1996) were excluded, based on our observation of the presence of 1 bp allele differences not found previously. Because of the number of alleles that differed by 1 bp at these two loci, we excluded them from the analysis. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstrap resamplings of the data, Algonquin Park and captive red wolves were grouped together in 72.4% of trees.



gray wolf making a significant contribution to the formation of the red wolf.

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

The historic Algonquin Park samples contained the C1 haplotype in 7 of 13 animals from which we were able to obtain control region sequences, and 9 of 12 red wolves contained the C2 haplotype.

The presence of the related C1 and C2 sequences in the geographically separated red wolves and eastern Canadian wolves but not in the Texas coyotes is consistent with a common origin of these two wolves. The remaining samples in this population contained coyote mtDNA sequences, confirming that some level of hybridization has also occurred.

The sequence divergence between the haplotypes observed in the eastern Canadian wolf and red wolf haplotype was 2.1%. The intraspecific sequence divergence for coyotes (*C. latrans*) was 1.7%. Comparisons indicated 3.2 % sequence divergence between the eastern Canadian wolf sequence (C1) and coyote sequences and 2.3 % sequence divergence between the red wolf (C2) and coyote haplotypes. The sequence divergence was approximately 8.0% between gray wolf (*C. lupus*) mtDNA and the haplotypes found in eastern Canadian wolves and red wolves, and 10.0% between gray wolf and coyote haplotypes. The sequence difference observed between eastern Canadian wolf sequences and coyote sequences is consistent with a separation of 150 000 – 300 000 years, using a divergence rate of 1–2% per 100 000 years for the mammalian control region (Stewart and Baker

Fig. 2. Neighbor-joining tree of Nei's genetic distances (1972) for allele frequencies from 8 microsatellite loci for eastern Canadian wolves, gray wolf populations, and a Texas coyote population. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. From the 1000 bootstraps resampling the data, the Algonquin Park and captive red wolf populations were grouped together in 67.8% of trees. The neighbor-joining tree gave an approximation of the genetic relationships among these populations, and alternative topologies are possible.

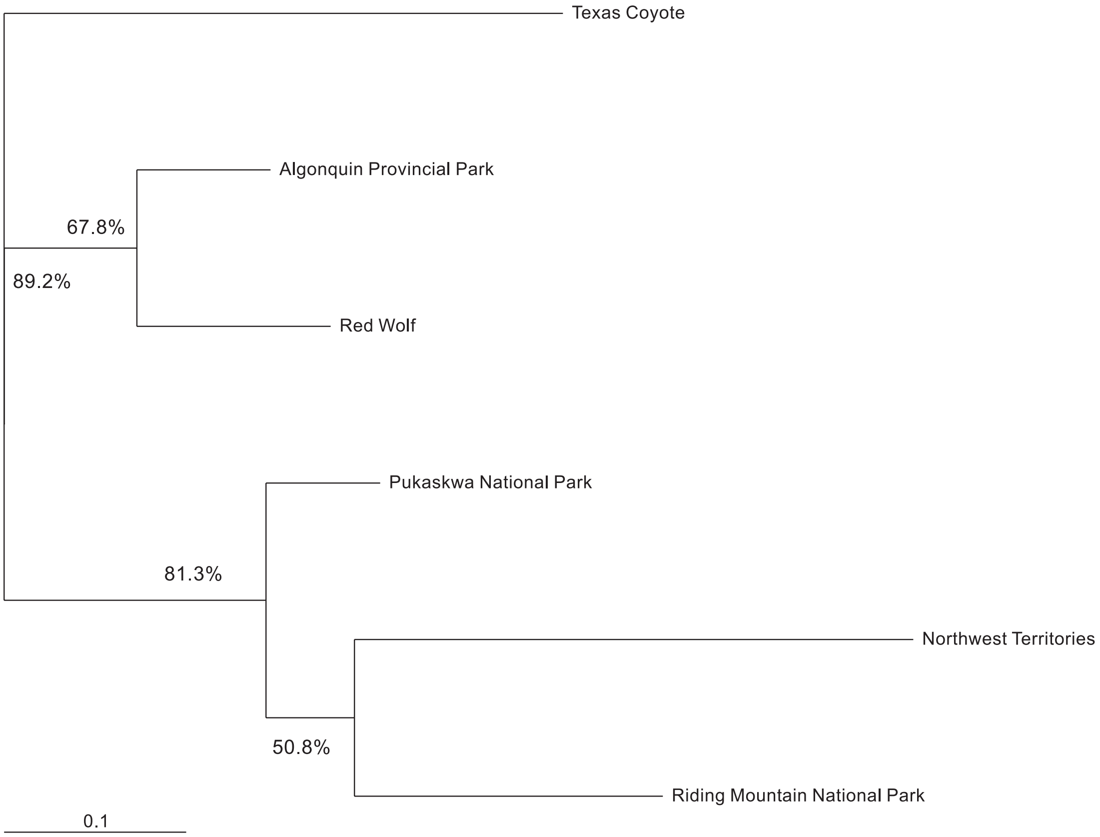


Table 1. Alleles prevalent in Texas coyotes and other coyote populations that are absent or present at low frequency among captive red wolves.

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

Note: Loci and allele designations were described previously (Roy et al. 1996).

1994), and is consistent with the 1–2 million year divergence between gray wolves and coyotes (Kurten and Anderson 1980; Wayne 1993; Vila et al. 1997).

Discussion

The similarity between the eastern Canadian wolf and the red wolf has been noted previously and both wolves were described as small eastern wolves long before the eastward expansion of coyotes occurred (Brewster and Fritts 1995). Neighbor-joining analysis of Nei's genetic distances using previously published data (Roy et al. 1994, 1996) and additional data we obtained from captive red wolf, other gray wolf, and coyote populations again grouped the eastern Canadian wolf population and captive red wolf samples. One interpretation of this relationship was that "hybridizing wolves" in Algonquin Park, southern Quebec, and Minnesota contained similar amounts of coyote genetic material to the red wolf (Roy et al. 1994). This interpretation did not seem consistent with the genetic distance between the red wolf and the Texas coyotes, which was the coyote source population closest to the red wolf. The absence of common coyote alleles in eastern Canadian wolves in Algonquin Park and the red wolf samples suggests that the close relationship observed between these two wolf populations was the result of a common wolf genetic origin. The application of assignment tests, I_I values, and POI values further supports the hypothesis that non-coyote-derived parts of the genome are responsible for the similarity between the red wolf and the eastern Canadian wolf.

The presence of distinct control region haplotypes within the eastern Canadian wolves from the historic Algonquin Park population, and the fact that captive red wolves clustered closer to coyotes than to gray wolves, support the evolution of the eastern wolves independently of the gray wolf. These data indicate that like the nuclear microsatellite DNA,

Fig. 3. (A) Log-likelihood individual indices (I_I) from captive red wolves (n = 60) and canids from Algonquin Park (n = 49) and Texas (n = 22). The I_I value was calculated for each individual animal DNA profile at 8 microsatellite loci, using the allele frequencies from the Algonquin Park population and the Texas coyote population, respectively. (B) A plot of the log probability of identity (POI) values for captive red wolves (n = 60) and Algonquin Park (n = 49) and Texas (n = 22) wolves, using the allele frequencies from the Algonquin Park population and the Texas coyote population, respectively.

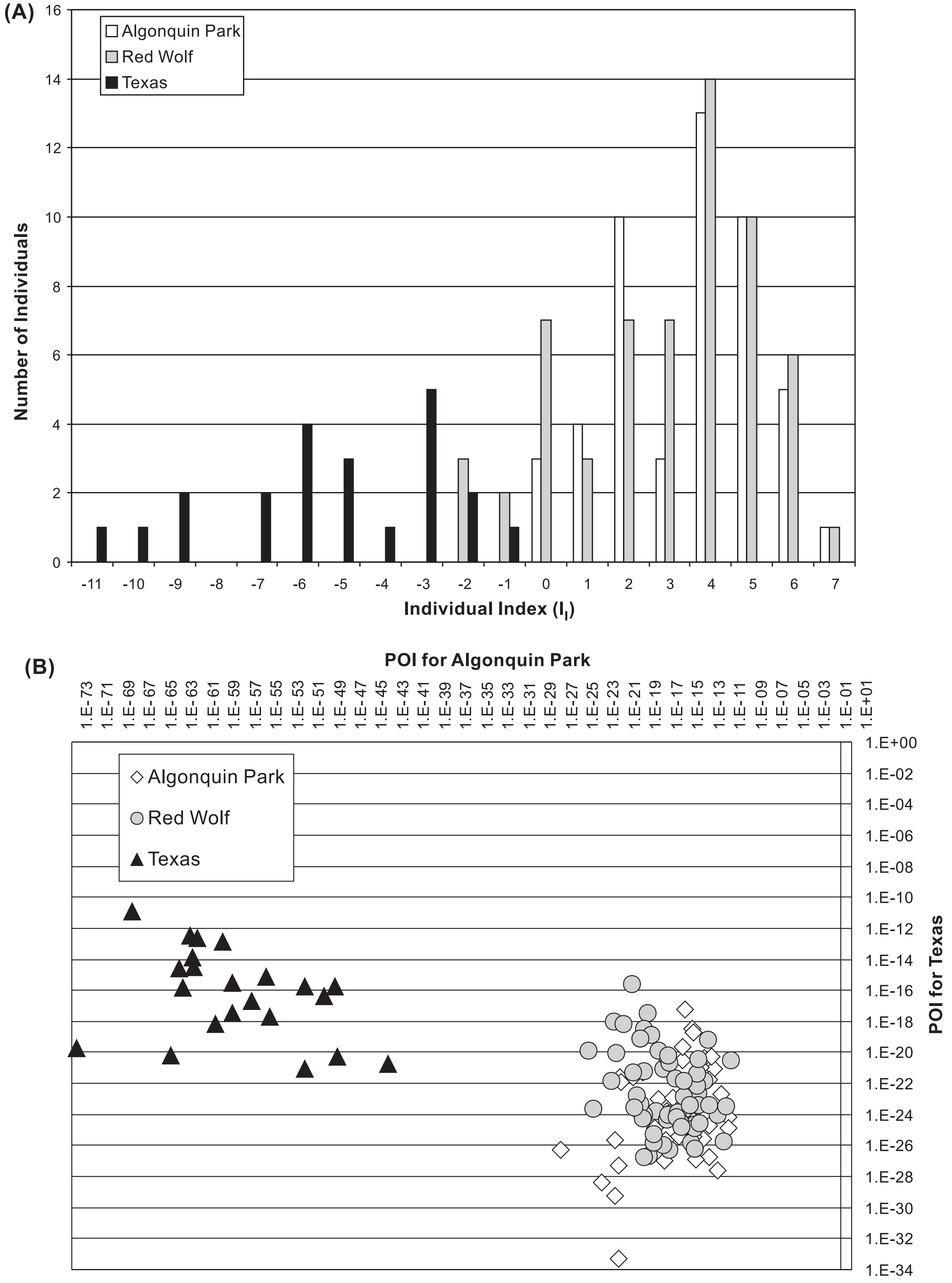


Fig. 4. (A) Plot of log-likelihood I_I values for captive red wolves (n = 60) and Algonquin Park (n = 49), Northwest Territories (N.W.T.) (n = 67), and Texas (n = 20) wolves. The I_I value was calculated for each individual animal DNA profile at 8 microsatellite loci, using the allele frequencies from the N.W.T. wolf population and the Texas coyote population, respectively. (B) A plot of the log of POI values from captive red wolves (n = 60) and Algonquin Park (n = 49), Northwest Territories (n = 67), and Texas (n = 22) wolves, using the allele frequencies from the Algonquin Park population and the N.W.T. population, respectively.

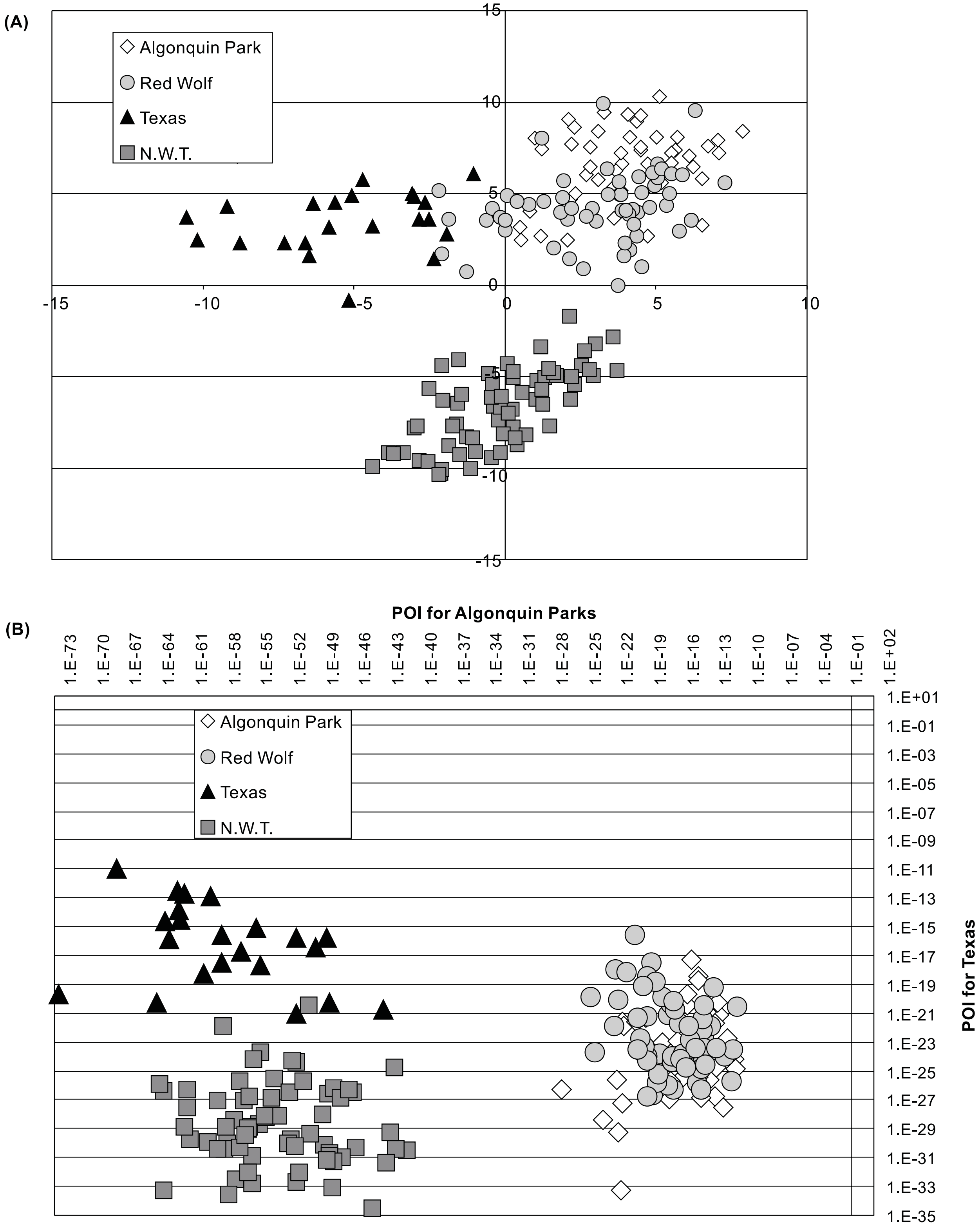


Fig. 5. (A) Minimum-spanning tree for 238 bp of control-region haplotypes from the red wolf, eastern Canadian wolf, and coyote. Sequences obtained from this study are given a C designation, e.g., Canis-1 (C1). Haplotypes found in eastern Canadian wolves (*C. lycaon*) are shaded and red wolf (*C. rufus*) haplotypes are hatched. The number of perpendicular lines between the haplotypes indicates the number of base-pair substitutions or insertions/deletions. (B) Neighbor-joining tree of sequence divergence for 238 bp of control-region haplotypes from the gray wolf, red wolf, eastern Canadian wolf, and coyote. The *lycaon/rufus* lineage has two nucleotides in the mtDNA control region common with *C. lupus* but different from *C. latrans*, which accounts for the proximity of *C. lupus* mtDNA to the *lycaon/rufus* haplotypes. The scale represents 0.100 or 10.0% sequence divergence. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped data sets. European wolf haplotypes (W1–W4) (Ellegren et al. 1996) are provided. Sample locations and corresponding haplotypes are as follows: red wolf captive breeding program: C2 (*n* = 9), C19 (*n* = 3); Algonquin Park and surrounding area (ca. 1960s): C1 (*n* = 7), C9 (*n* = 1), C14 (*n* = 3), C17 (*n* = 1), C19 (*n* = 1); southern Ontario (ca. 1960s): C1 (*n* = 1), C9 (*n* = 1), C14 (*n* = 2), C19 (*n* = 4); north of Algonquin Park (ca. 1960s): C1 (*n* = 1), C16 (*n* = 1), C23 (*n* = 1); northern boreal region of Ontario (ca. 1960s): C23 (*n* = 1); northwestern Ontario (ca. 1960s): C13 (*n* = 2), C24 (*n* = 1); Manitoba: C3 (*n* = 1), C22 (*n* = 1), C23 (*n* = 1); Ohio: C5 (*n* = 1); Texas: C4 (*n* = 1), C6 (*n* = 2), C7 (*n* = 1), C8 (*n* = 1), C10 (*n* = 1), C11 (*n* = 1), C12 (*n* = 1), C15 (*n* = 1), C18 (*n* = 2), C19 (*n* = 12), C20 (*n* = 2), C21 (*n* = 2); northern Quebec: C23 (*n* = 1), Northwest Territories: C23 (*n* = 1), Fort Francis, Ontario: C23 (*n* = 1).

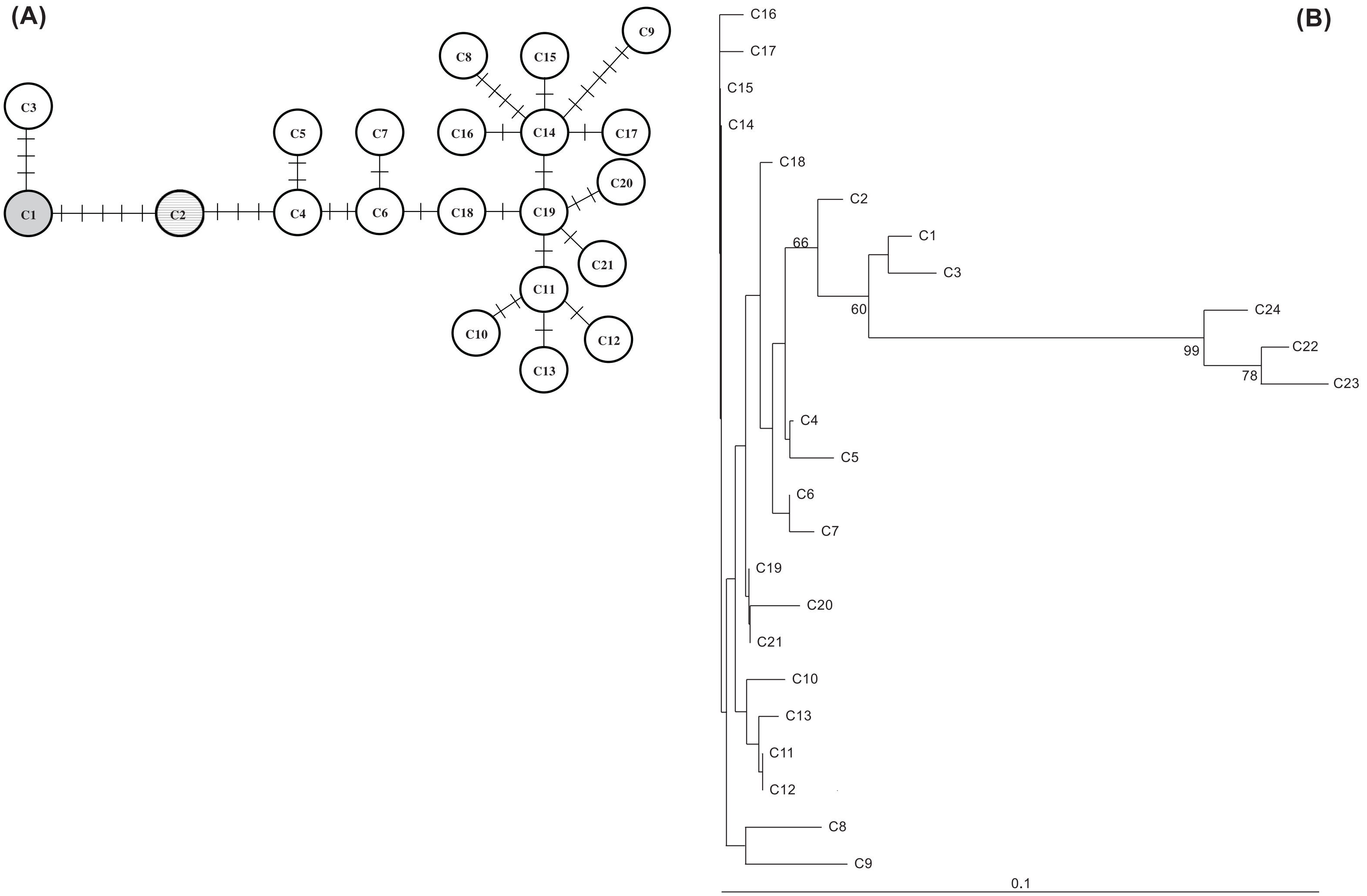
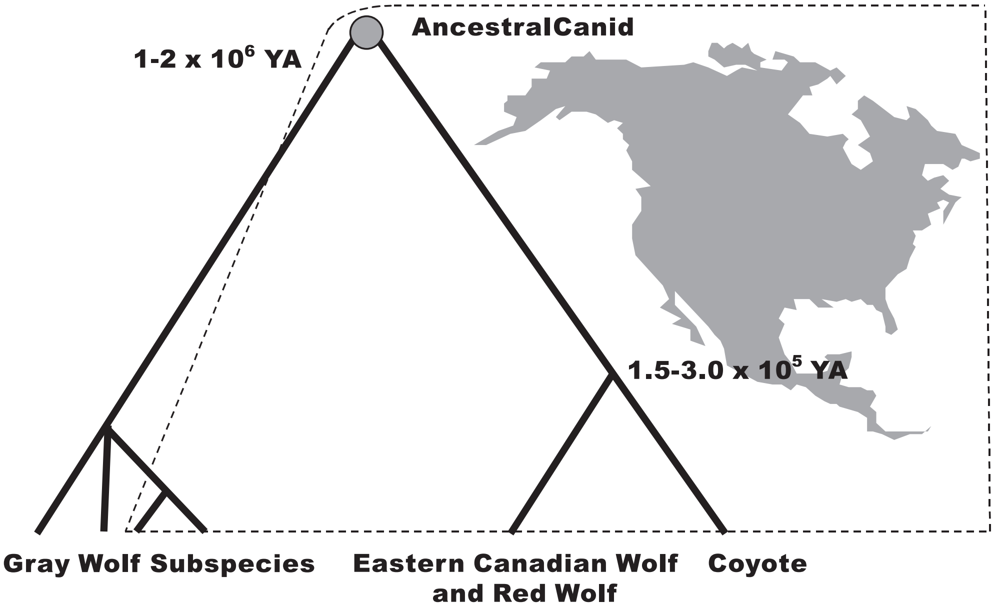


Fig. 6. A model of the evolution of North American wolves. The progenitor of *C. lupus*, *C. lycaon*, and *C. latrans* is indicated at the top. It is generally accepted that divergence from this ancestor occurred 1–2 million years ago (YA), when the progenitor of *C. lupus* migrated to Eurasia. The North American species diverged 150 000 – 300 000 years ago into the eastern Canadian wolf/red wolf (*C. lycaon*) and the coyote (*C. latrans*). Recently, *C. lycaon* and *C. latrans* have come into contact and have subsequently hybridized. The Eurasia-evolved *C. lupus* returned to North America within the Pleistocene.



the mtDNA of the eastern Canadian wolf and red wolf is not of gray wolf origin but is similar to that of coyotes because of their relatively recent divergence from a common ancestor. It is unlikely that the eastern Canadian wolf mtDNA haplotypes obtained from the early 1960s samples represent the total introgression of coyote mtDNA, as the Algonquin Park population would have had only 30 years of contact with the expanding coyote population, and would require the replacement of gray wolf (*C. lupus*) mtDNA.

The coyote has been identified as the canid species that evolved in the New World (Nowak 1979; Wayne 1993). Our data indicate that the North American canid mtDNA lineage diverged into (1) the red wolf and eastern Canadian wolf and (2) the coyote. We propose a model (Fig. 6) in which these two lineages diverged within the late Pleistocene, 150 000 – 300 000 years ago, and came into contact during post-settlement time as a result of extensive habitat alteration. Further, North American wolves and coyotes evolved independently of the gray wolf, *C. lupus*, which evolved in Eurasia 1–2 million years ago. We suggest that the eastern North American wolf adapted to catching prey such as white-tailed deer within a forested habitat and the western coyote adapted to arid regions and smaller prey. The red wolf mtDNA haplotype, while showing sequence similarity to eastern Canadian wolf mtDNA, is less divergent from coyote mtDNA and this may reflect continued contact with coyotes.

Several lines of evidence support a common origin for the red wolf and eastern Canadian wolf: (1) the historic range of the eastern Canadian wolf overlaps that of the present-day red wolf, and both would have existed in southern refugia during the Pleistocene (Nowak 1979; Brewster and Fritts 1995); (2) Pleistocene fossils suggest that a small wolf inhabited eastern North America (Nowak 1995); (3) species that evolved in the New World and diverged only 150 000 – 300 000 years ago are more likely to have hybridized with each other than with the gray wolf. The lack of introgression of coyote DNA into western gray wolves and even Mexican gray wolves, *C. l. baileyi* (Lehman et al. 1991; Garcia-Moreno et al. 1996; Roy et al. 1996), populations sympatric with coyotes, suggests that eastern Canadian wolves and red wolves are the only wolves that hybridize readily with coyotes. The fact that the Mexican wolf shows no hybridization with coyotes suggests that the smaller size of the eastern wolves is not the reason for their hybridization with coyotes.

The predisposition of the eastern North American wolves to hybridize with coyotes may represent an evolutionary characteristic unique to these wolves, and suggests that the red wolf (*C. rufus*) and the eastern Canadian wolf (*C. l. lycaon*) have a common origin. Several additional lines of evidence are consistent with the hypothesis of a common origin for these wolves. Comparisons of skull morphology indicate similarities between *C. rufus* and *C. l. lycaon* (Lawrence and Bossert 1967, 1975; Nowak 1979, 1995). Algonquin Park wolves were previously described as a remnant red wolf population, classified at the time as *Canis niger* (Stanfield 1970). A common origin has also been suggested by Mech (1971), who stated “if the red wolf is a hybrid between the wolf and coyote, it would be this sub-species (*C. l. lycaon*) of wolf that is involved.”

The only evidence contrary to the hypothesis of a North American-evolved wolf is the apparent presence of gray wolf (*C. lupus*) mtDNA haplotypes within 6 red wolf samples collected from the southeastern U.S.A. and samples from the northwestern Great Lakes region. *Canis lupus* mtDNA haplotypes were identified in 3/6 (Wayne and Jenks 1991) and 3/11 pre-1940s (Roy et al. 1996) red wolves from the historical range of *C. rufus*. We question whether these 6 samples were red wolves, as the historic range of *C. rufus* has been identified as overlapping the distribution of the gray wolf subspecies *C. l. nubilus* (Caire et al. 1989; Schwartz and Schwartz 1991) and a Texas range of *C. l. baileyi* (Nowak et al. 1995). Gray wolf mtDNA was also found in 16% of the 77 previously analyzed animals (Wayne and Jenks 1991) from the region where they were selected for the breeding program. Strict morphological criteria were used to classify the animals as red wolf, coyote, or red wolf – coyote hybrid, and 44 were selected. Subsequent selection of the most representative red wolf types provided 17 animals that were used as founders. We suggest that the 12 animals with gray wolf mitochondrial DNA from the original 77 may have been of *C. l. nubilus*, *C. l. baileyi*, or *C. l. familiaris* origin.

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

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

In summary, much of the debate between Nowak and Wayne concerning the red wolf has focused on the presence of coyote genetic material in red wolves (Nowak 1992; Wayne et al. 1992, 1998; Nowak and Federoff 1998). However, the main issue stems from the claim that gray wolf mtDNA occurs in red wolves and eastern Canadian wolves. It is generally accepted that the gray wolf, *C. lupus*, evolved in Eurasia (Nowak 1979; Wayne 1993; Vila et al. 1997). Nowak has proposed that a coyote-like progenitor originating in North America diverged on two continents, evolving independently into the red wolf and the gray wolf. If a wolf evolved in North America, its mtDNA should be more similar to that of coyotes, *C. latrans*, than to that of gray wolves, *C. lupus*, and this was observed in historic eastern Canadian wolves and in the captive red wolf program. Wayne's hypothesis is that gray wolves and coyotes hybridized to form the red wolf. The support for this hypothesis was the absence in red wolves of distinct genetic markers not found in coyotes or gray wolves. We have identified a group of mtDNA control region sequences more closely related to those of coyotes than those of gray wolves that are specific to the red wolf and eastern Canadian wolf (Fig. 5). The mtDNA data support the microsatellite data that indicate a close relationship between the red wolf, *C. rufus*, and eastern Canadian wolf, *C. l. lycaon*. Furthermore, the absence of gray wolf mtDNA and the distribution of assignment test scores away from the gray wolf distribution in captive red wolves and eastern Canadian wolves support the evolution of a small North American wolf independently of the gray wolf. The data presented lead to the formal rejection of the hypothesis that the red wolf and eastern Canadian wolf are hybrids of coyotes and gray wolves. Furthermore, we also reject the hypothesis that the eastern Canadian wolf is a subspecies of the gray wolf. At present the red wolf exists as the species *C. rufus*; however, based on historical taxonomic classifications, the eastern North American wolves would require the classification *C. lycaon* (Brewster and Fritts 1995).

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

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