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## Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of gray wolf origin

P.J. Wilson, S. Grewal, T. McFadden, R.C. Chambers, and B.N. White

**Abstract:** We analysed the mitochondrial DNA (mtDNA) from two historical samples of eastern North American wolves: the last wolf reported to have been killed in northern New York State (ca. 1890s) and a wolf killed in Maine in the 1880s. These wolves represent eastern wolves, presently classified as the gray wolf (*Canis lupus*) subspecies *Canis lupus lycaon*, which were present well before the expansion of western coyotes (*Canis latrans*) into these regions. We show the absence of gray wolf mtDNA in these wolves. They both contain New World mtDNA, supporting previous findings of a North American evolution of the eastern timber wolf (originally classified as *Canis lycaon*) and red wolf (*Canis rufus*) independently of the gray wolf, which originated in Eurasia. The presence of a second wolf species in North America has important implications for the conservation and management of wolves. In the upper Great Lakes region, wolves of both species may exist in sympatry or interbreed with each other, which impacts the accuracy of estimates of numbers of wolves of each species within this geographic region. Furthermore, the historical distribution of the eastern timber wolf (*C. lycaon*), as revealed by these skin samples, has important implications for the reintroduction of wolves into the northeastern U.S. states, such as New York and Maine.

**Résumé :** Nous avons analysé l'ADN mitochondrial (ADNm) dans des échantillons recueillis chez deux loups « historiques » de l'est de l'Amérique du Nord, le dernier loup signalé dans le nord de l'état de New York (ca. 1890s) et un loup tué dans le Maine dans les années 1880. Ces deux loups représentent les loups de l'est reconnus actuellement comme appartenant à une sous-espèce (*Canis lupus lycaon*) du loup gris (*Canis lupus*) présente dans ces régions bien avant l'expansion de l'aire de répartition du coyote de l'ouest (*Canis latrans*). Nous démontrons ici l'absence d'ADNm de loup gris chez ces deux loups. Ils contiennent tous les deux de l'ADNm de loup du Nouveau Monde, ce qui confirme la découverte antérieure de l'évolution en Amérique du Nord du loup des bois de l'est (classifié à l'origine sous le nom de *Canis lycaon*) et du loup roux (*Canis rufus*), indépendamment de celle du loup gris d'origine eurasiennne. La présence d'une deuxième espèce de loup en Amérique du Nord a des conséquences importantes pour la conservation et la gestion des loups. Dans la région supérieure des Grands Lacs, les loups des deux espèces peuvent vivre en sympatrie et se croiser, ce qui affecte l'exactitude des estimations du nombre de loups de chaque espèce dans cette région géographique. De plus, la répartition passée du loup des bois de l'est (*C. lycaon*), telle que révélée par l'analyse de ces échantillons de peau, a des conséquences importantes sur la réintroduction des loups dans les états du nord-est américain comme le New York et le Maine.

[Traduit par la Rédaction]

### Introduction

All of eastern North America was historically inhabited by wolves: the eastern timber wolf (also known as the east-

ern Canadian wolf), currently designated a subspecies of the gray wolf (*Canis lupus*), *Canis lupus lycaon*, in the north, and the red wolf, *Canis rufus*, in the south. Both are smaller than the gray wolves found in more western and northern re-

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gions and are taxonomically distinct from western coyotes (*Canis latrans*) (Lawrence and Bossert 1967, 1975; Mech 1971; Nowak 1979, 1995; Schmitz and Kolenosky 1985). Hybridization has been identified between eastern timber wolves and coyotes (Lawrence and Bossert 1967, 1975; Mech 1971; Schmitz and Kolenosky 1985; Lehman et al. 1991; Wayne and Lehman 1992; Roy et al. 1994), and it has been suggested that *C. rufus* is not a valid species but the result of recent extensive hybridization between gray wolves and coyotes (Wayne and Jenks 1991; Roy et al. 1996).

Recent genetic evidence demonstrates a close relationship between the eastern timber wolf (also called the eastern wolf) and the red wolf consistent with both being North American-evolved species (Wilson et al. 2000; Hedrick et al. 2002). In a study of the mitochondrial DNA (mtDNA) control region of eastern wolves present in Ontario in the 1960s and captive red wolves, DNA sequences that were 150 000 – 300 000 years divergent from those found in western coyotes were identified. These sequences and DNA profiles at microsatellite loci have led to the proposal that the eastern wolf diverged from the western coyote about 150 000 – 300 000 years ago. It is generally accepted that the gray wolf evolved in Eurasia (Old World) and came to North America approximately 300 000 years ago, and is 1–2 million years divergent from the western coyote (Kurtén and Anderson 1980; Wayne 1993; Vila et al. 1997).

To test the hypothesis of a North American-evolved wolf, we analysed samples from wolves that were present in eastern North America prior to the proposed arrival of western coyotes. Two historical skin samples collected from New York State (ca. 1890s) and Maine (ca. 1880s) provided by the Adirondack Museum, Blue Mountain Lake, N.Y., and the Museum of Comparative Zoology, Harvard University, Cambridge, Mass., respectively, were used to test this hypothesis. Following a 40-year period during which few, if any, large wild canids were known to occur in New York after the State offered a bounty, a coyote-like animal was reported in the St. Lawrence valley in 1920. Reports of coyote-like animals continued to increase in the early 1930s in Ontario and the first report in Maine was made in 1936 (Moore and Parker 1992). Based on the absence of coyotes during the period when these wolves were taken, the first prediction is that if the eastern timber wolf is truly a subspecies of the gray wolf, then prior to the arrival of coyotes, the mtDNA would be of *C. lupus* origin. The second prediction is that if the eastern timber wolf is a North American-evolved wolf more closely related to the coyote, the mtDNA sequences would be closely related to those of *C. latrans* as a result of evolutionary history and not recent hybridization (Wilson et al. 2000).

## Materials and methods

### Sample collection

The Maine historical sample (1880s) (Harvard Museum Accession No. 50518) had been reported as a wolf collected by bounty in Penobscot County. The historical New York sample (ca. 1890s) from the Adirondack Museum has been described as the last wolf killed within the State. Based on observation, the mounted New York wolf demonstrates a wolf-like morphology. Both hide samples were extracted us-

ing a modified QIAmp (Qiagen) extraction protocol with a lysis buffer 1× lysis buffer (4 M urea, 0.2 M NaCl, 0.5% *n*-lauroyl sarcosine, 10 mM CDTA (1,2-cyclohexanediamine), 0.1 M Tris-HCl, pH 8.0).

### Control-region sequencing and sequence analysis

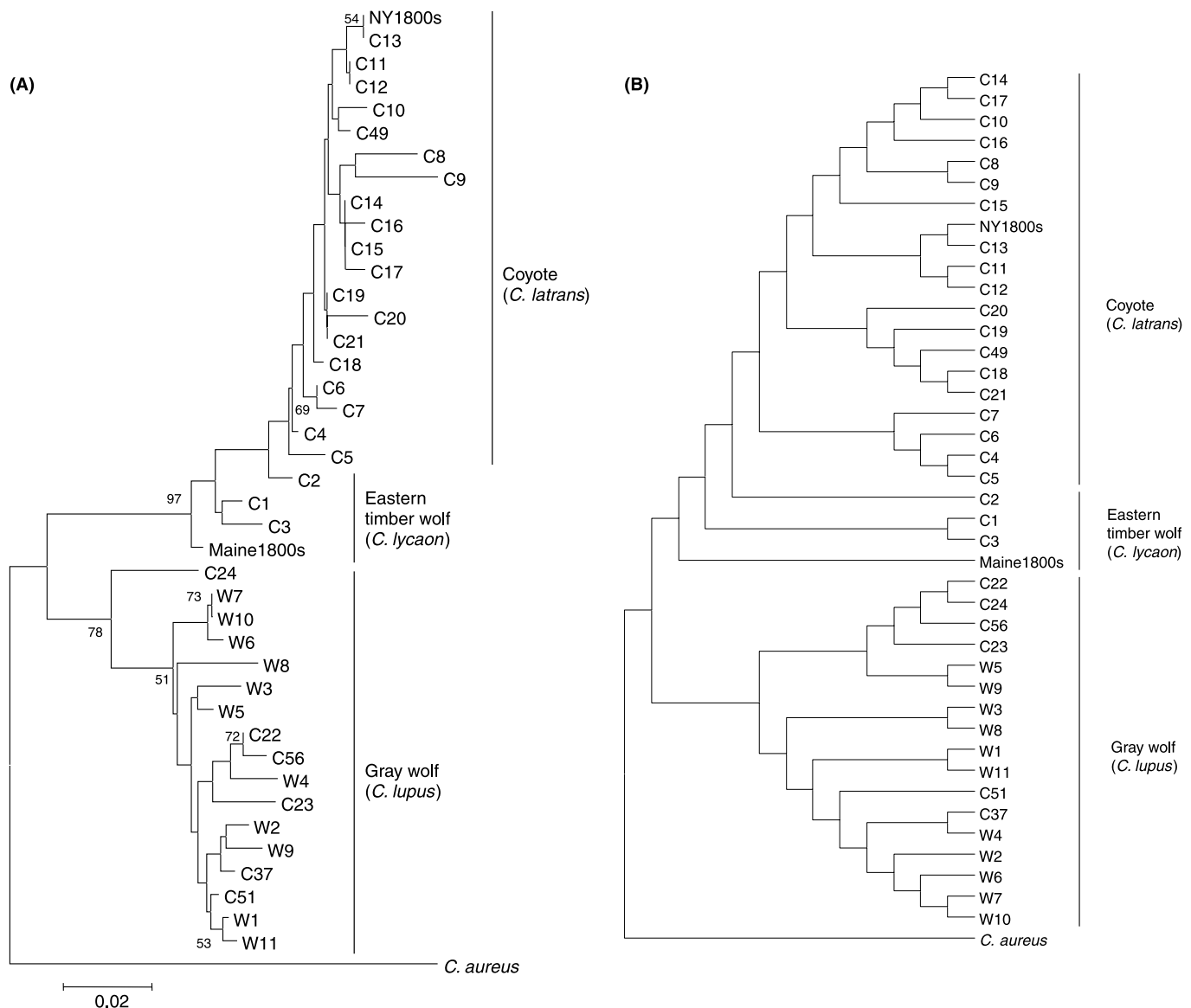
The following primers were used to amplify the control region of the mtDNA: primer 1: 5'-GAA GCT CTT GCT CCA CCA ATC-3' (Pilgrim et al. 1998); primer 2: 5'-GGG CCC GGA GCG AGA AGA GGG AC-3'. The control region was amplified in a total reaction volume of 20 µL per tube using 25 ng of genomic DNA, 200 µM dNTPs, 1× amplification buffer, 2.0 mM MgCl<sub>2</sub>, 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 reamplified and purified through QIAquick (Qiagen) for DNA sequencing using dye-terminator cycle sequencing using an ABI Prism 373 DNA sequencer. The phylogenetic relationships of canid mtDNA haplotypes were generated using a neighbour-joining tree with Kimura's two-parameter DNA distance (transition-to-transversion ratio 2:1) using the MEGA software package (Kumar et al. 2002) and 1000 bootstraps of the sequence data. A maximum-parsimony analysis was also performed on the sequences using MEGA (Kumar et al. 2002), using close-neighbour interchange, search level 3. Both analyses incorporated complete deletions among the sequence comparisons.

## Results and discussion

We were successful in generating approximately 230 base pairs (bp) of mtDNA control region sequence for the two historical samples in a region previously described in Pilgrim et al. (1998) and Wilson et al. (2000). Both the Maine sample and the New York State specimen contained a sequence that was not of gray wolf origin. A diagnostic insertion/deletion (indel) had previously been identified to distinguish coyote and gray wolf control region sequences (Pilgrim et al. 1998). The two historical sequences showed a pattern consistent with coyote-like sequences that excluded a gray wolf mtDNA based on the above diagnostic sequence. This was consistent with the clustering of these two sequences with coyote and eastern wolf sequences and not the gray wolf sequences (Fig. 1). Furthermore, the Maine sample contained a mtDNA with a sequence that clustered with previously identified eastern wolf- and red wolf-specific mtDNA sequences using both Kimura's DNA distance and a neighbour-joining-tree construction (Fig. 1A) and maximum parsimony (Fig. 1B). The mtDNA control region of the New York hide sample did not cluster with this group (Wilson et al. 2000), but clustered with sequences found in modern western coyotes (Fig. 1).

In the absence of gray wolf mtDNA, the possible explanations for these results include (i) the early movement, i.e., 40 years prior to recorded sightings, of coyotes into New York State and Maine followed by hybridization with local eastern wolves, and (ii) a common evolutionary ancestry of coyotes and the North American-evolved eastern wolf (Wilson et al. 2000; Hedrick et al. 2002), resulting in similar mtDNA sequences. The first explanation, early coyote hybridization,

**Fig. 1.** Phylogenetic analysis of haplotypes representing approximately 230 bp of the control-region mtDNA from grey wolf, coyote, eastern timber wolf, red wolves and the two historic sequences from Maine and New York State. The following *Canis* mtDNA haplotypes were included: European wolf haplotypes (W1–W4) (Ellegren et al. 1996), W5–W10 (GenBank Accession Nos. AF33807–12), and W11 (AF487754). Additional sample locations and corresponding haplotypes are as follows: red wolf captive-breeding program: C2 ( $n = 9$ ; the matriline haplotypes of the captive red wolf program have been confirmed subsequent to Wilson et al. 2000); 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 ( $n = 2$ ), C23 ( $n = 3$ ), C37 ( $n = 1$ ); 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: C22 ( $n = 1$ ), C23 ( $n = 1$ ); Northwest Territories: C22 ( $n = 8$ ), C23 ( $n = 1$ ), C51 ( $n = 1$ ), C56 ( $n = 1$ ); Fort Francis, Ontario: C23 ( $n = 1$ ); Florida: C49 ( $n = 1$ ). (A) A neighbor-joining tree of Kimura's two-parameter DNA distance measure (2:1 transition-to-transversion ratio) for an approximately 230-bp region of coyote, grey wolf, eastern timber wolf, red wolf and golden jackal (*Canis aureus*) control-region haplotypes. Bootstrap values are provided for nodes that were observed in greater than 50% of 1000 bootstrapped datasets. (B) Maximum-parsimony analysis of canid mtDNA haplotype sequences.



cannot be excluded on the basis of the New York mtDNA sequence, given its similarity to coyote sequences. However, the similarity of the Maine sequence to mtDNA found in eastern timber wolves from Algonquin Provincial Park in the 1960s and at present, as well as that obtained from the

red wolf captive-breeding program (Fig. 1) (Wilson et al. 2000), supports the presence of a wolf that is not of gray wolf (*C. lupus*) origin. For both the New York and Maine wolves to contain "coyote" mtDNA would require two independent hybridization events with coyotes that migrated into

these states 40 and 50 years, respectively, before any recorded sightings (Moore and Parker 1992).

Although the presence of a coyote-like sequence in the New York wolf could have resulted from coyotes entering the area and hybridizing with remnant eastern wolves, this finding does not exclude its origin as a North American-evolved eastern timber wolf (*C. lycaon*). Wolf-coyote hybridization appears to be limited to eastern North America, based on field observations (Paquet 1989, 1991, 1992; Thurber and Peterson 1991) and genetic studies (Lehman et al. 1991; Wayne and Lehman 1992; Roy et al. 1994; Pilgrim et al. 1998). This eastern distribution of hybridization corresponds to the historical distributions of the eastern timber wolf and red wolf (Nowak 1979, 1995; Brewster and Fritts 1995). While it does not appear that the gray wolf and coyote readily interbreed, there is emerging evidence that the eastern timber wolf and gray wolf can hybridize (Mech and Federoff 2002).

We interpret the absence of gray wolf mtDNA in these two historical samples and the similarity of the Maine control-region sequence to previously identified eastern timber wolf – red wolf sequences as supporting the presence of an eastern wolf with an evolutionary history independent of that of the gray wolf. This result does not support the present classification of the eastern timber wolf as a subspecies of the gray wolf (*C. l. lycaon*) but indicates that it is a North American-evolved wolf more closely related to the coyote (Wilson et al. 2000; Hedrick et al. 2002) that was previously proposed to be reclassified as *C. lycaon* (Wilson et al. 2000), based on its original taxonomic consideration (Brewster and Fritts 1995).

The original presence of a North American-evolved wolf in large areas of Canada and the U.S.A. has significant conservation ramifications for management programs and proposed reintroduction efforts. Areas may contain two different species of wolves, the eastern timber wolf and the gray wolf, and it is presently unclear to what extent they might interbreed. We are presently examining the levels of interbreeding between *C. lupus* and *C. lycaon*. Conservation and management of wolves in North America depends on an assessment of population sizes and this can only be made when the species are clearly identified.

In addition, the presence of the eastern timber wolf, *C. lycaon*, within Maine and New York in the 1880s has implications for reintroduction efforts in terms of which wolf species to move into these states. The genetic evidence supports the reintroduction of the eastern timber wolf, based on historical distribution. However, the feasibility of this will depend on the risk of hybridization with the existing eastern coyote (Paquet et al. 1999). Only two historical samples from these states have been analysed to date, and other historical specimens may reveal the presence of gray wolves, *C. lupus*, within such areas as the north woods of Maine, suggesting the feasibility of reintroducing them into these regions.

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