

Native Great Lakes wolves were not restored

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Wolves from the Great Lakes area were historically decimated due to habitat loss and predator control programmes. Under the protection of the US Endangered Species Act, the population has rebounded to approximately 3000 individuals. We show that the pre-recovery population was dominated by mitochondrial DNA haplotypes from an endemic American wolf referred to here as the Great Lakes wolf. In contrast, the recent population is admixed, and probably derives also from the grey wolf (*Canis lupus*) of Old World origin and the coyote (*Canis latrans*). Consequently, the pre-recovery population has not been restored, casting doubt on delisting actions.

Keywords: hybridization; coyote; introgression; aDNA

1. INTRODUCTION

Habitat modification can have both positive and negative effects on the abundance and distribution of species. A striking example in this regard is the distribution of two native American carnivorans: the grey wolf (*Canis lupus*) and the coyote (*Canis latrans*). Habitat loss and direct persecution led to the eradication of grey wolves over most of the contiguous USA and parts of southern Canada (Carbyn 1987). With the extinction of wolves and associated habitat changes, the smaller coyotes expanded their geographical range from the central plains and western North America north to Alaska, east to the Atlantic and south to Panama (Hilton 1978; Beckoff 1982; also see Sacks *et al.* 2004).

Owing to protection under the US Endangered Species Act, the restoration of wolves to the Great Lake states of Minnesota, Wisconsin and Michigan has been a remarkable success. The population was nearly exterminated approximately 100 years ago but now numbers over 3000 individuals, an increase which has led to a recent delisting action (Fish and Wildlife Service 2003). The delisting petition defined this wolf as *Canis lupus lycaon*, a subspecies of grey wolf. However, the petition also acknowledged controversy concerning the taxonomic status of Great Lakes wolves and the likelihood that hybridization between wolf-like taxa (the western grey wolf, *C. lupus*; the Great Lakes wolf, *C. l. lycaon* and the coyote, *C. latrans*) in the area had obscured genetic distinction. Consequently, a

critical concern is the degree to which restoration has preserved the pre-recovery form of wolf that inhabited the US Great Lake states and whether delisting favours the persistence of hybrids at the expense of pure forms. These concerns are further complicated by the recent suggestion based on molecular genetic data that *C. l. lycaon* is a distinct wolf-like species unique to the region that evolved independently in North America from a coyote-like ancestor. Consequently, hybridization among three wolf-like species is a possibility (Lehman *et al.* 1991; Wilson *et al.* 2000; Kyle *et al.* 2006).

2. MATERIAL AND METHODS

We genetically analysed 17 historic wolves approximately 100 years old and 68 recent wolves representing the diversity of coyote-like ($n=54$) and wolf-like ($n=14$) haplotypes identified in Canadian and US samples by Lehman *et al.* (1991; figure 1 and table 1). DNA was extracted from historic specimens with phenol-chloroform as in Leonard *et al.* (2005). Precautions for the analysis of low-copy DNA include nucleic acid isolation in separately designated ancient DNA facility, negative controls and replications. Two PCR and extraction blanks were run with each PCR to monitor for contamination. We used three different sets of primers to produce overlapping sequences for comparison to detect contamination (Leonard *et al.* 2002). Further, 26 fragments were replicated twice, 9 fragments three times and 1 fragment four times. One historic haplotype was also observed in modern specimens and two historic haplotypes were found in multiple specimens, suggesting that polymerase error was not the origin of these haplotypes. Recent samples were amplified as in Vilà *et al.* (1999).

Average sequence divergence within and between taxa and their standard errors, as well as bootstrap replicates, were assessed using MEGA (Kumar *et al.* 2004). Population size of the historic population was estimated from nucleotide diversity (θ) using DNASP v. 4.10.7 (Tajima 1996; Rozas & Rozas 1999). We estimated female effective population size from the expression $\theta=2N_{ef}\mu$, where N_{ef} is the female effective population size and μ is the mutation rate. We used a value of μ based on a divergence time between coyotes and grey wolves of 2 million years (Nowak 2003) and a generation time of 3 years (Mech & Seal 1987), which yields a μ of 1.1×10^{-7} substitutions/site/generation. Consequently, a census size was estimated assuming equal reproduction of the sexes and that 30% of the population were reproductive adults (Vilà *et al.* 1999). A divergence rate of 2μ was used with the average HKY distances between Great Lakes area wolf haplotypes to estimate their age of origin.

Phylogenetic trees were based on 421–422 base pair (bp) sequences of the 5' end of the mitochondrial control region from pre-recovery Great Lakes wolves, recent Great Lakes wolves and previously published North American grey wolf and coyote sequences (Vilà *et al.* 1999; Leonard *et al.* 2005). Three methods were used to construct the phylogeny. First, a neighbour-joining phylogeny was constructed in PAUP* v. 4.0.b10 (Swofford 2002) using a HKY model of sequence evolution with a gamma shape parameter $\alpha=0.0683$, estimated in MODELTEST v. 3.04 (Posada & Crandall 1998). The support for the internal nodes was estimated by 1000 bootstrap pseudoreplicates. Second, maximum parsimony trees using a heuristic search and indels as a fifth state were constructed using PAUP* v. 4.0.b10. Finally, Bayesian analyses using MRBAYES v. 3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) were repeated twice for 3 500 000 steps, at which point the standard deviation of split frequencies was less than 0.01, and the first 25% of trees were discarded as burnin.

Departure from monophyly of the Great Lakes wolves was examined in MACCLADE v. 3.06 (Maddison & Maddison 2002) by determining the number of additional steps required to force monophyly of the pre-recovery Great Lakes wolf haplotypes (excluding GL4; see §3). The likelihood of the most parsimonious tree and one in which the pre-recovery Great Lakes wolves (except GL4) were monophyletic were calculated in PAUP* v. 4.0.b10.

3. RESULTS

Comparison of pre-recovery Great Lakes wolf sequences show that, with the exception of one Quebec wolf (haplotype GL4), all haplotypes are most closely related to each other and divergent from sequences in extant grey wolves and coyotes.

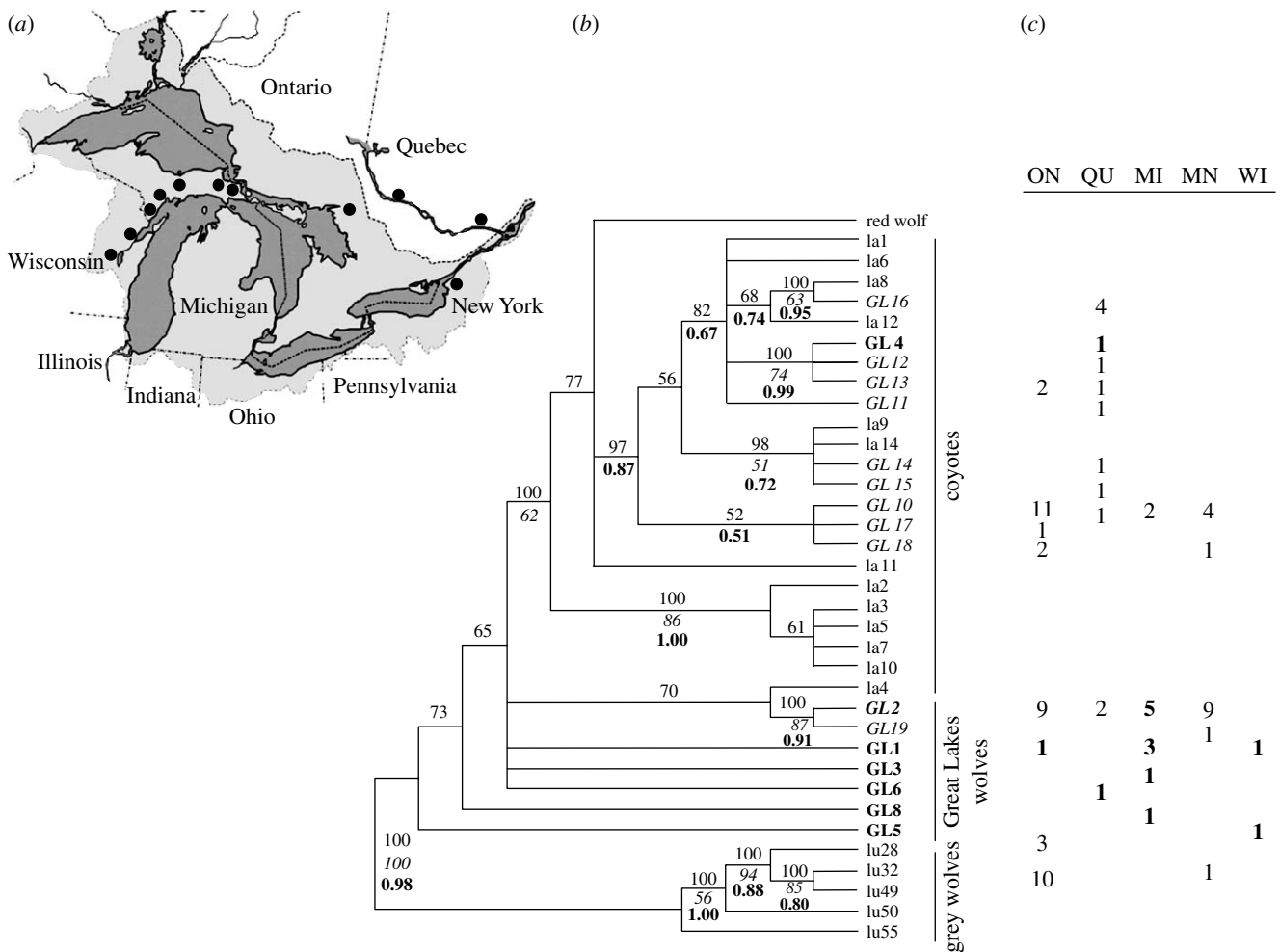


Figure 1. (a) The map of the Great Lakes area was modified from the US Army Corps of Engineers, Detroit District map (<http://gis.glin.net/maps/>). Approximate localities of historic specimens are indicated with filled circles. (b) Parsimony consensus cladogram of Great Lakes wolves (GL, historic in bold and recent in italics), North American grey wolves (lu), red wolf (*C. rufus*) and coyotes (la). The percentage of the most parsimonious trees with a given node is indicated above branches. Bootstrap support of neighbour-joining phylogeny is indicated in italics and Bayesian posterior probability is shown in bold. (c) The number of observations of each Great Lakes wolf haplotype in Ontario (ON), Quebec (QU), Michigan (MI), Minnesota (MN) and Wisconsin (WI) with historic samples in bold.

Table 1. Historic wolf samples from the National Museum of Natural History, Smithsonian Institution. (Collection number, date, locality and mitochondrial DNA haplotypes are indicated.)

museum number	year	locality	haplotype
USNM 178452	1910 ^a	Algonquin Park, Ontario, Canada	GL1
USNM 140561	1905	Mattawa, Quebec, Canada	GL6
USNM 140562	1905	Mattawa, Quebec, Canada	GL7 ^b
USNM 223171	1916	Quebec City, Quebec, Canada	GL4
USNM 148897	1906	Marquette Co., Michigan, USA	GL1 ^b
USNM 148898	1906	Marquette Co., Michigan, USA	GL1
USNM 168820	1909	Calderwood, Michigan, USA	GL8
USNM 168821	1909	Calderwood, Michigan, USA	GL1
USNM 170566	1910	Hulbert, Michigan, USA	GL2
USNM 170567	1910	Taquahmenon River, Michigan, USA	GL2
USNM 170621	1910	Taquahmenon River, Michigan, USA	GL2
USNM 170692	1910	Cusino, Michigan, USA	GL2
USNM 171132	1911	Sault Sainte Marie, Michigan, USA	GL2
USNM 180798	1912	Dickinson Co., Michigan, USA	GL3
USNM A01804	^c	Adirondacks, New York, USA	GL9 ^b
USNM 150421	1907	Eagle River, Wisconsin, USA	GL5
USNM 156838	1908	Taylor Co., Wisconsin, USA	GL1

^a Approximation.
^b Sequence incomplete.
^c Specimen accessioned before 1893.

Excluding haplotype GL4, pre-recovery sequences differ by 2–12 bps (0.5–4.5%) from each other and by an average of 13.6 ± 3.8 (s.d.) ($6.4 \pm 2.9\%$) and 24.7 ± 2.5 bp ($19.1 \pm 5.1\%$) from coyotes and grey wolves, respectively. There are 49 or 60 parsimony informative sites in the dataset used to construct the phylogeny, depending on inclusion of indels as informative sites.

Phylogenetic analysis with the grey wolf as out-group suggests that pre-recovery Great Lakes wolf sequences are basal to those in modern coyotes (figure 1). A tree that constrains all pre-recovery sequences to be monophyletic is only one step longer than the shortest tree and not less likely. We estimated female effective population size of historic Great Lakes wolves to be approximately 60 000 from the observed value of $\theta = 0.013$. This implies a census size of approximately 400 000 individuals, far greater than the current population size. Based on the average divergence among pre-recovery haplotypes excluding GL4, this diversity probably originated approximately 300 000 years ago.

We compared pre-recovery sequences with those in recent Great Lakes wolves and found that only haplotype GL2 persists (figure 1). Haplotype GL1 may also be extant as it is identical to a shorter sequence identified in recent Ontario wolves (haplotype C1; Wilson *et al.* 2000). Additionally, a haplotype more closely related to the pre-recovery haplotypes than to coyotes was identified in Minnesota (GL19). Haplotypes GL1 and GL19 were found in 21 of 68 (31%) of the recent Great Lakes wolves that we typed. The other 47 samples had grey wolf ($n = 14$, 21%) or coyote haplotypes ($n = 33$, 49%; figure 1). Consequently, the mitochondrial DNA data suggest that the dominant wolf currently existing in the Great Lakes area is derived principally from coyotes, grey wolves and their hybrid descendants, with a smaller contribution from the pre-recovery Great Lakes wolf. However, nuclear DNA analyses are needed to further confirm this conclusion.

4. DISCUSSION

Our results show that the wolves inhabiting the Great Lakes states prior to recovery were genetically distinct and had a large geographical range. Comparison of pre- and post-recovery Great Lakes wolves suggests that phylogenetically distinct wolf-like canids largely supplanted the pre-recovery form. These new invaders have a mixed ancestry including contributions from grey wolves, coyotes and Great Lakes wolves. Prior genetic evidence suggests that hybridization has been increasing in the Great Lakes region over the past 50 years and that hybrids now populate large areas of the Great Lakes states and New England (Lehman *et al.* 1991; Roy *et al.* 1994; Wilson *et al.* 2000; Kyle *et al.* 2006). Historical records indicate that hybridization between coyotes and wolf-like canids in the area did not begin until after 1900 when coyotes first entered the region (Hilton 1978; Lehman *et al.* 1991). The coyote invasion was probably a consequence of habitat alteration, changes in prey abundance, reduction in wolf numbers and other anthropogenic causes.

If hybridization is a result of natural factors, such as post-glacial range expansion, and involves entities listed by the Endangered Species Act, then hybrids can justifiably be protected (Jenks & Wayne 1992; Fish and Wildlife Service 1996). Indeed, hybrids may provide material for adaptation and evolution (Arnold 1997) and lead to increased population viability (Hedrick 1995). However, if hybridization is occurring in disturbed habitats and otherwise reflects human activities that can be mitigated, then delisting should be postponed to protect the listed entity until such contributing factors are ameliorated (Jenks & Wayne 1992). The US Endangered Species Act protects distinct populations (Pennock & Dimmick 1997) and hybrids (Fish and Wildlife Service 1996). Therefore, protection of the population in the US Great Lakes can be justified given that the historical conditions that have given rise to the current population can be sufficiently understood.

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- Arnold, M. L. 1997 *Natural hybridization and evolution*. Oxford, UK: Oxford University Press.
- Beckoff, M. 1982 Coyote. In *Wild mammals of North America: biology, management, and economics* (eds J. A. Chapman & G. A. Feldhamer), pp. 447–459. Baltimore, MD: Johns Hopkins University Press.
- Carbyn, L. N. 1987 Gray wolf and red wolf. In *Wild furbearer management and conservation in North America* (eds M. Novak, J. A. Baker, M. E. Obbard & B. Malloch), pp. 359–376. Toronto, Canada: Ministry of Natural Resources.
- Fish and Wildlife Service 1996 Proposed rules. *Federal Register* **61**, 4709–4713.
- Fish and Wildlife Service 2003 Proposed rules. *Federal Register* **68**, 15 876–15 879.
- Hedrick, P. W. 1995 Gene flow and genetic restoration: the Florida panther as a case study. *Conserv. Biol.* **9**, 996–1007. (doi:10.1046/j.1523-1739.1995.9050996.x)
- Hilton, H. H. 1978 Systematics and ecology of the eastern coyote. In *Coyotes: biology, behavior, and management* (ed. M. Bekoff), pp. 209–228. New York, NY: Academic Press.
- Huelsenbeck, J. P. & Ronquist, F. 2001 MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755. (doi:10.1093/bioinformatics/17.8.754)
- Jenks, S. M. & Wayne, R. K. 1992 Problems and policy for species threatened by hybridization: the red wolf as a case study. In *Wildlife 2001: populations* (eds D. R. McCullough & R. H. Barrett), pp. 237–251. London, UK: Elsevier Science.
- Kumar, S., Tamura, K. & Nei, M. 2004 MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**, 150–163. (doi:10.1093/bib/5.2.150)
- Kyle, C. J., Johnson, A. R., Patterson, B. R., Wilson, P. J., Shami, K., Grewal, S. K. & White, B. N. 2006 Genetic nature of eastern wolves: past, present and future. *Conserv. Genet.* **7**, 273–287. (doi:10.1007/s10592-006-9130-0)
- Lehman, N., Eisenhauer, A., Hansen, K., Mech, D. L., Peterson, R. O., Gogan, P. J. P. & Wayne, R. K. 1991

- Introgression of coyote mitochondrial DNA into sympatric North American gray wolf populations. *Evolution* **45**, 104–119. (doi:10.2307/2409486)
- Leonard, J. A., Wayne, R. K., Wheeler, J., Valadez, R., Guillén, S. & Vilà, C. 2002 Ancient DNA evidence for Old World origin of New World dogs. *Science* **298**, 1613–1616. (doi:10.1126/science.1076980)
- Leonard, J. A., Vilà, C. & Wayne, R. K. 2005 Legacy lost: genetic variability and population size of extirpated US gray wolves (*Canis lupus*). *Mol. Ecol.* **14**, 9–17. (doi:10.1111/j.1365-294X.2004.02389.x)
- Maddison, D. R. & Maddison, W. P. 2002 *MACCLADE*, version 4. Sunderland, MA: Sinauer Associates.
- Mech, L. D. & Seal, U. S. 1987 Premature reproductive activity in wild wolves. *J. Mammal.* **68**, 871–873. (doi:10.2307/1381570)
- Nowak, R. M. 2003 Wolf evolution and taxonomy. In *Wolves: behavior, ecology, and conservation* (eds L. D. Mech & L. Boitani), pp. 239–258. Chicago, IL: University of Chicago Press.
- Pennock, D. S. & Dimmick, W. W. 1997 Critique of the evolutionarily significant unit as a definition for “distinct population segments” under the U. S. Endangered Species Act. *Cons. Biol.* **11**, 611–619.
- Posada, D. & Crandall, K. A. 1998 MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818. (doi:10.1093/bioinformatics/14.9.817)
- Ronquist, F. & Huelsenbeck, J. P. 2003 MRBAYES3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574. (doi:10.1093/bioinformatics/btg180)
- Roy, M. S., Geffen, E., Smith, D., Ostrander, E. & Wayne, R. K. 1994 Patterns of differentiation and hybridization in North American wolf-like canids revealed by analysis of microsatellite loci. *Mol. Biol. Evol.* **11**, 553–570.
- Rozas, J. & Rozas, R. 1999 DNASP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174–175. (doi:10.1093/bioinformatics/15.2.174)
- Sacks, B. N., Brown, S. K. & Ernest, H. B. 2004 Population structure of California coyotes corresponds to habitat-specific breaks and illuminates species history. *Mol. Ecol.* **13**, 1265–1275. (doi:10.1111/j.1365-294X.2004.02110.x)
- Swofford, D. L. 2002 *PAUP* : phylogenetic analysis using parsimony (*and other methods)*, version 4. Sunderland, MA: Sinauer Associates.
- Tajima, F. 1996 The amount of DNA polymorphism maintained in a finite population when the neutral mutation rate varies among sites. *Genetics* **143**, 1457–1465.
- Vilà, C., Amorim, I. R., Leonard, J. A., Posada, D., Castroviejo, J., Petrucci-Fonseca, F., Crandall, K. A., Ellegren, H. & Wayne, R. K. 1999 Mitochondrial DNA phylogeography and population history of the gray wolf *Canis lupus*. *Mol. Ecol.* **8**, 2089–2103. (doi:10.1046/j.1365-294x.1999.00825.x)
- Wilson, P. J. *et al.* 2000 The relationship of the red wolf and eastern Canadian wolf provides evidence for a common evolutionary history, independent of the gray wolf. *Can. J. Zool.* **78**, 2156–2166. (doi:10.1139/cjz-78-12-2156)