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

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

Keywords: hybridization; mitochondrial haplotype; microsatellite genotype; eastern wolf

1. INTRODUCTION

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

Recently, Leonard & Wayne (2008) have reported on mitochondrial DNA (mtDNA) analyses of the historic GLR wolves, suggesting that pre-recovery wolves were dominated by haplotypes distinct from grey wolves (*C. lupus*) and western coyotes (*C. latrans*) which they propose are from an endemic North American wolf referred to as the 'Great Lakes wolf'. They interpreted the current population to be admixed, deriving primarily from *lupus/latrans* hybridization, with minor contributions from the Great

Lakes wolf, and concluded that recently delisted GLR wolves are not genetically representative of the pre-recovery population. Their interpretation fails to recognize extensive genetic data on the NW evolved eastern wolf (*C. lycaon*), the mtDNA sequences of which are close to those of *C. latrans* (Wilson *et al.* 2000, 2003). The eastern wolf has been shown to be a distinct species (Wilson *et al.* 2000) that is capable of hybridizing with both coyotes and grey wolves across its range (see Kyle *et al.* 2006), acting as the conduit of *Canis* hybridization in northeastern North America. We do not agree with the suggestion that the current GLR population contains animals derived from *lupus/latrans* hybridization, as these sympatric species do not hybridize in western North America (see Kyle *et al.* 2006).

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

2. MATERIAL AND METHODS

We extracted DNA from three historic *Canis* samples provided by the University of Wisconsin Zoological Museum (table 1) using a DNeasy Blood & Tissue Kit (Qiagen Inc., Mississauga, ON) in a dedicated ancient DNA laboratory. A 343–347 bp fragment of the mtDNA control region was amplified using the primers described in Wilson *et al.* (2003) and conditions similar to theirs, except that we included 0.1 μg/μl BSA in the reaction and used an annealing temperature of 60°C. Contamination was monitored during extraction and PCR using negative controls. PCR products were cleaned with ExoSAP-IT (USB Corporation, Cleveland, OH) prior to sequencing on a MegaBACE 1000 (GE Healthcare). The sequence of sample 11 856 was confirmed from an independent amplification. The sequences were edited, aligned and compared with known haplotypes in BIOEDIT (Hall 1999). Refer to Wilson *et al.* (2000, 2003) for a description of the sequences. Amplification of eight nuclear microsatellite loci was attempted for each sample (Wilson *et al.* 2000), and homozygous genotypes were confirmed by repeated amplification. Two samples were genotyped at eight loci and the remaining sample at six loci.

Alleles were scored in GENEMARKER (v. 1.7, SoftGenetics LLC 2004) and the data were analysed using STRUCTURE (v. 2.2, Pritchard *et al.* 2000), including samples from studies by Grewal (2001) and Wilson *et al.* (submitted): Northwest Territories ($n=67$); Manitoba ($n=41$); Minnesota ($n=9$); northwestern Ontario ($n=30$); northeastern Ontario ($n=34$); Algonquin Provincial Park ($n=49$); Frontenac Axis ($n=74$); Adirondacks ($n=66$); Saskatchewan ($n=36$); and Texas ($n=24$). The admixture model of STRUCTURE was run for $K=1$ to $K=10$ with five repetitions of 10^6 iterations following a burn-in period of 250 000 iterations for each K. The number of populations K was determined to be five, based on the criteria outlined by Pritchard *et al.* (2000) and Evanno *et al.* (2005). The three historic samples were assigned a proportional membership to each of the five genetic clusters.

To supplement the results from STRUCTURE, a non-model-based factorial correspondence analysis (FCA) was performed on the microsatellite data for individual canids using GENETIX (v. 4.05, Belkhir *et al.* 2004). Two-factorial components FC-1 and FC-2, which accounted for 6.35 and 3.61 per cent of the total inertia, respectively, were plotted to visualize the clustering of the historic samples in relation to the other sample groups.

3. RESULTS

We compared the informative variable approximately 230 bp region of the Great Lakes wolf mtDNA control region haplotypes within Leonard & Wayne (2008), denoted as GL(X), to those within Wilson *et al.* (2000, 2003), denoted as C(X). As previously identified by Leonard & Wayne (2008), haplotype

Table 1. mtDNA haplotypes and admixture proportions of historic *Canis* samples.

museum catalogue no.	sex	state	county	date	haplotype	no. of loci	admixture proportions				
							P1	P2	P3	P4	P5
8626	M	MN	Itasca	Spring 1900	C13	8	0.009	0.012	0.180	0.791	0.008
8627	M	MN	Itasca	February 1899	C13	6	0.027	0.027	0.351	0.564	0.030
11 856	–	WI	Ashland	Winter 1907/08	C1	8	0.020	0.056	0.250	0.618	0.057

Table 2. Comparison of variable sites between GL(X) and C(X) haplotypes: dot indicates same base as the uppermost row; dash indicates no base present. (Superscripts indicate identical haplotypes within approximately 230 bp region of comparison. Note C(X) haplotypes do not span entire alignment of GL(X) haplotypes.)

haplotype	variable site within GL(X) haplotypes														
	100	159	170	230	231	232	247	249	253	264	265	266	268	271	301
GL1[a]	A	C	C	T	C	C	T	G	C	T	T	C	C	A	T
GL2 and GL19[b]	·	T	·	·	·	·	C	·	·	C	·	·	·	·	·
GL10, GL17 and GL18[c]	G	·	·	·	T	T	C	A	T	·	·	·	·	·	C
GL11[d]	G	·	–	C	·	T	C	A	T	·	·	·	·	·	C
GL12[e]	G	·	·	C	·	T	C	A	·	·	·	·	·	G	C
GL13[f]	G	·	·	C	·	T	C	A	T	·	·	·	·	G	C
GL16[g]	G	·	–	·	·	T	C	A	T	C	C	T	T	G	C
C1[a]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
C3[b]	·	T	·	·	·	·	C	·	·	C	·	·	·	·	·
C9[g]	G	·	–	·	·	T	C	A	T	C	C	T	T	G	C
C13[c]	G	·	·	·	T	T	C	A	T	·	·	·	·	·	C
C14[f]	G	·	·	C	·	T	C	A	T	·	·	·	·	G	C
C17[e]	G	·	·	C	·	T	C	A	·	·	·	·	·	G	C
C19[d]	G	·	–	C	·	T	C	A	T	·	·	·	·	·	C

GL1 was identical to *C. lycaon* haplotype C1. However, we found other similarities among haplotypes from both studies (table 2), including two GL(X) haplotypes identical to *C. lycaon* haplotype C3. It is of interest that three GL(X) haplotypes were identical to a coyote-clustering sequence, haplotype C13, which has not been found in extant coyote populations but is present throughout the distribution of *C. lycaon* (Grewal 2001).

We sequenced the three historic samples at the mtDNA control region (294–322 bp) and assigned the haplotypes based on the approximately 230 bp region (table 1). The two haplotypes we observed in the three samples, C13 ($n = 2$) and C1 ($n = 1$), were identical to haplotypes found by Leonard & Wayne (2008) (table 2).

Based on the genotypes at the microsatellite loci, five groups were identified by STRUCTURE: Texas and Saskatchewan (western coyotes) = P1; Frontenac Axis and Adirondacks (eastern coyotes) = P2; Algonquin (eastern wolves) = P3; Manitoba, Minnesota and northwestern/northeastern Ontario (eastern/grey wolves) = P4; and Northwest Territories (grey wolves) = P5. The admixture proportions of the three historic samples revealed that their highest proportional memberships were to the P4 and P3 groups (table 1). The individual-based FCA clustered the historic samples among the Manitoba, Minnesota, northwestern/northeastern Ontario and Algonquin clusters (figure 1).

4. DISCUSSION

The genetic analyses show that the three historic samples from the western GLR have a mixed ancestry deriving primarily from groups representing eastern and grey wolves (i.e. P3 and P4). The historic samples did not cluster significantly with either of the two groups composed of coyote-like animals (i.e. P1 and P2). The results of the FCA were concordant with the results from STRUCTURE with the historic samples clustering with wolves and not coyotes. Both OW and NW mtDNA haplotypes occur in P3 and P4 (Grewal 2001), whereas only OW haplotypes occur in P5 (Wilson *et al.* 2003) and only NW haplotypes occur in P1 and P2 (Wilson *et al.* submitted) (figure 1). Given that OW and NW haplotypes occur in the groups for which the historic samples had their highest proportional memberships, we conclude that the historic samples represent animals containing genetic material derived from both grey (OW) and eastern wolves (NW), and not coyotes.

The occurrence of haplotype C13 in two historic samples that clustered with non-coyote groups based on nuclear microsatellite data (table 1) supports our interpretation that C13 is a *C. lycaon* haplotype, as does its apparent absence from extant coyote
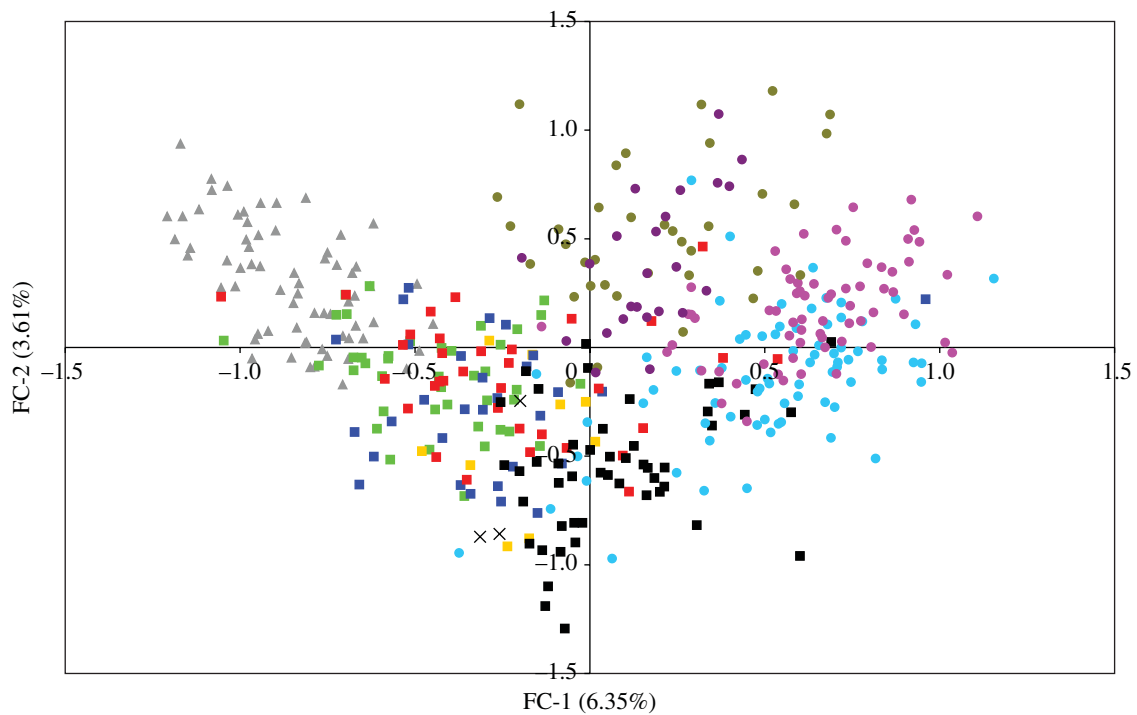
Figure 1. FCA of microsatellite loci for *Canis* sample groups. Symbol type of sample group indicates mtDNA haplotypes present: squares, OW and NW (bright green, MB; gold, MINN; dark blue, NWON; red, NEON; black, ALG); triangles, OW (grey, NWT); circles, NW (sky blue, FRAX; pink, ADIR; dark yellow, SASK; violet, TXS); crosses, HIST.
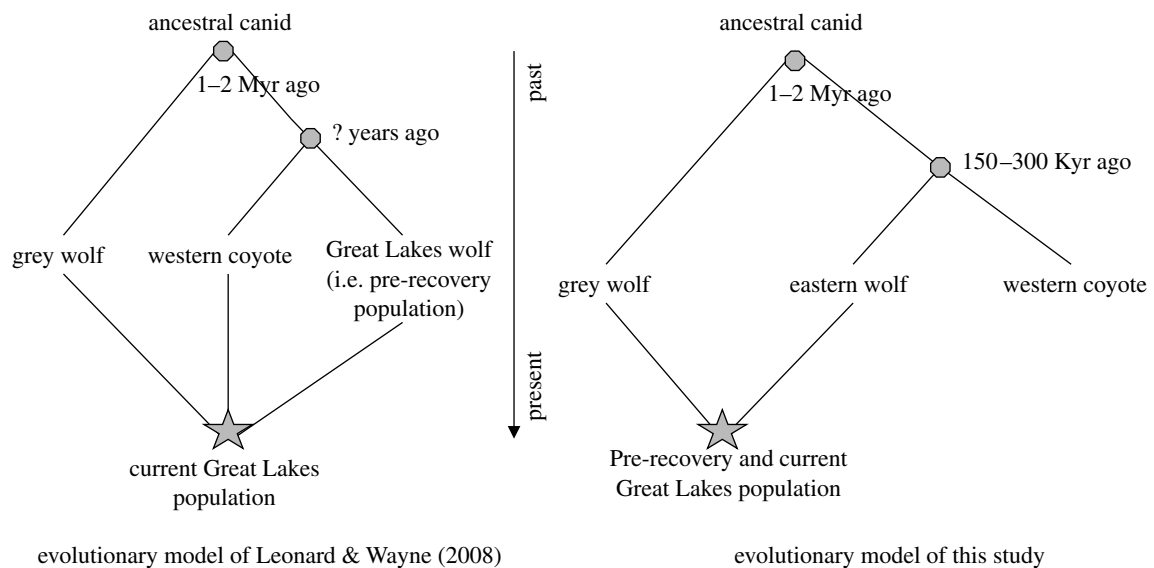

Figure 2. Comparison of *Canis* evolution hypotheses. Star, hybridization; circle, divergence.

populations in the regions with no evidence of wolf–coyote hybridization, specifically Texas and Saskatchewan (Wilson *et al.* submitted, data not shown). The occurrence of haplotype C13 in wolves 100 yr ago is probably the result of one of three possible scenarios: (i) C13 evolved in the common ancestor of coyotes and eastern wolves and was perpetuated in both species when they diverged (i.e. incomplete lineage sorting), (ii) *lycaon/latrans* hybridization occurred earlier, i.e. pre-European settlement, whereby C13 was introgressed into *C. lycaon* and subsequently lost from the source *C. latrans* population, and (iii) an ancestral coyote haplotype was introgressed into the *C. lycaon* lineage during the

Pleistocene or sometime prior to European settlement and subsequently diverged to become eastern wolf specific. The latter scenario would explain why C13 clusters closer to coyote sequences than eastern wolf sequences (Wilson *et al.* 2003). The loss of an mtDNA haplotype from a source *C. latrans* population seems unlikely, given the rapid population expansion of the species and the apparent absence of C13 from non-hybridizing coyote populations. The divergence of haplotype C13 from the eastern wolf clade and its absence in coyote populations (Wilson *et al.* 2003) supports C13 as being of eastern wolf origin through introgressive hybridization and subsequent divergence, and not incomplete lineage sorting.

The GL(X) haplotypes that were identical to haplotypes C1, C3 and C13 (table 2) occurred in samples from the western Great Lakes states (Leonard & Wayne 2008), further supporting the presence of *C. lycaon* genetic material in animals in this region.

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

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

We suggest that recolonizing wolves originating from Minnesota, Manitoba and northwestern Ontario, containing *C. lupus* and *C. lycaon* mtDNA haplotypes, moved east into Wisconsin and continued into Michigan's Upper Peninsula. This may have resulted in the current wolf populations exhibiting *C. lupus* mtDNA in higher frequency than in the pre-recovery wolves from the western GLR. Previous research supports the presence of both *C. lycaon* and *C. lupus* haplotypes in Manitoba and northwestern Ontario (Wilson *et al.* 2003), which represents a potential and likely source of immigrants for the recovering Wisconsin and Michigan populations. Recent genetic analysis of *Canis* samples from the western GLR, based on a variety of genetic markers, supports *lupus/lycaon* hybridization (Wheeldon, unpublished data), as does previous research (Mech & Federoff 2002). The hypothesis that the current Great Lakes wolf population is derived from *lupus/latrans* hybridization is rejected by the data (figure 2).

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

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