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RESEARCH ARTICLE

Genetic and morphometric analysis of sixteenth century *Canis* skull fragments: implications for historic eastern and gray wolf distribution in North America

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Abstract Resolving the taxonomy and historic ranges of species are essential to recovery plans for species at risk and conservation programs that aim to restore extirpated populations. In eastern North America, planning for wolf population restoration is complicated by the disputed historic distributions of two wolf species: the Old World-evolved gray wolf (*Canis lupus*) and the New World-evolved eastern wolf (*C. lycaon*). We used genetic and morphometric data from 4- to 500-year-old *Canis* samples excavated in London, Ontario, Canada to help clarify the historic range of these two wolf species in the eastern temperate forests of North America. We isolated DNA and sequenced the mitochondrial control region and found that none of the samples were of gray wolf origin. Two of the DNA sequences corresponded to those found in present day coyotes (*C. latrans*), but morphometric comparisons show an eastern wolf, not coyote, origin. The remaining two sequences matched ancient domestic dog haplotypes. These results suggest that the New World-evolved eastern wolf, not the gray wolf, occupied this region prior to the arrival of European settlers, although eastern-gray wolf hybrids cannot be ruled out. Furthermore, our data support the idea of a shared common ancestry between eastern wolves and western coyotes, and that the distribution of gray wolves at this time probably did not include the eastern temperate forests of North America.

Keywords Eastern wolf · Morphometrics · Mitochondrial DNA · Phylogenetics · Precontact North America · Wolf restoration

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Introduction

Conservation programs aimed at restoring extirpated populations generally have poor outcomes (Frankham et al. 2004), primarily because the scientific information required for recovery is lacking (Seddon et al. 2007; Armstrong and Seddon 2008). Some recent restoration efforts, however, included substantial monitoring that has provided new insights for this approach. For example, the restoration of gray wolves (*Canis lupus*) to Yellowstone National Park is one of the most successful reintroduction projects ever undertaken (Smith et al. 2003; VonHoldt et al. 2008). This success, combined with new knowledge on the positive ecological effects experienced under top-down regulation by apex predators (Terborgh et al. 2001; Sergio et al. 2005; Chapron et al. 2008; Wallach et al. 2009), are likely to rekindle interest in wolf restoration in other parts of North America.

One of the main priorities for restoration biology is that feasibility studies include molecular genetic research to confirm the taxonomic status and historic range of candidate species (IUCN 1998; Armstrong and Seddon 2008). In eastern Canada and the United States (US), however, the presence of two distinct wolf species, the Old-World

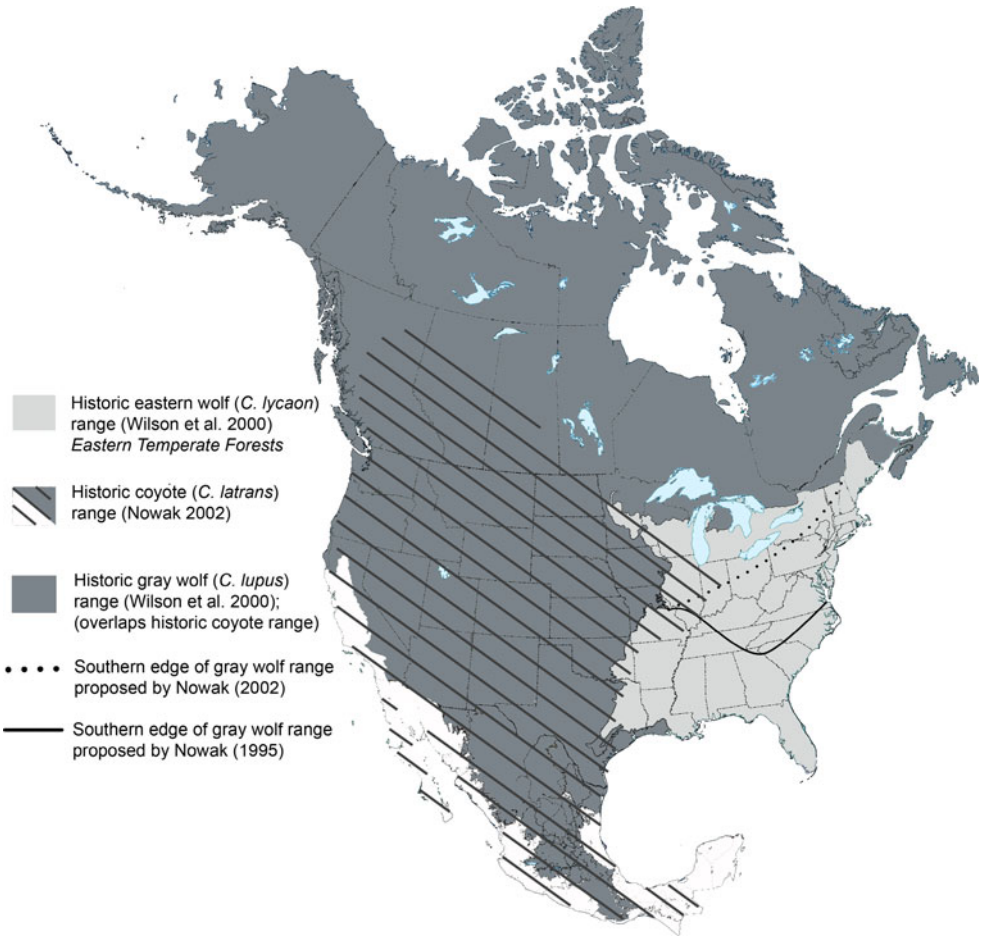

Fig. 1 Map of historic distribution of *Canis* spp. based on various taxonomic treatments. Historic eastern wolf (*C. lycaon*) range is in the Eastern Temperate Forest ecoregion and includes the red wolf (*C. rufus*) range shown south of the *thin solid line* (Wilson et al. 2000; Kyle et al. 2006); historic coyote (*C. latrans*) range shown in central and western North America (Nowak 2002; Sacks et al. 2004); historic gray wolf range overlapped historic coyote range (Nowak 2002); two suggested southern limits of *C. lupus lycaon* are also shown (*solid thin line* (Nowak 1995) and *dotted line* (Nowak 2002)). Current coyote (*C. latrans*) distribution is continent-wide (Moore and Parker 1992)

evolved gray wolf (*C. lupus*) and the New World-evolved eastern wolf (*C. lycaon*) (Wilson et al. 2000, 2003; Grewal et al. 2004; Kyle et al. 2006, 2008) brings a level of complexity to wolf restoration that is not experienced in western North America. In addition, extensive hybridization of eastern wolves with both gray wolves and expanding western coyotes (*C. latrans*) (Wheeldon and White 2009; Wilson et al. 2009) that were historically limited to areas of central and western North America (Fig. 1) (Nowak 2002; Sacks et al. 2004) until the early twentieth century (Hilton 1978; Nowak 1979; Kurtén and Anderson 1980), further complicates efforts to reconcile historic ranges of wolves in the east, as does the ongoing debate regarding the influence and timing (recent, historic, and/or ancient) of coyote and gray wolf hybridization with eastern wolves (Leonard and Wayne 2008, 2009; Wheeldon and White 2009; Mech 2009; Koblmüller et al. 2009). In fact, plans to restore gray wolves to Adirondack Park, New York were temporarily suspended, in part because the historic presence of gray wolves in the area was questioned (Paquet et al. 1999). More specifically, genetic evidence demonstrated a close relationship between eastern wolves and the endangered red wolves (*C. rufus*) of the southeastern US (Wilson et al. 2000), and mitochondrial DNA sequences found in late nineteenth century samples from New York state and Maine were not of gray wolf (Old World) origin (Wilson et al. 2003). This genetic evidence suggested a different historic distribution of gray wolves than that proposed by Nowak (1995, 2002) based solely on morphometric analyses (Fig. 1).

Although genetic analysis of ancient DNA is becoming a prominent tool for resolving wildlife conservation issues including historic distributions (Leonard 2008), the limited availability of historic *Canis* samples leaves the question of what wolf species originally inhabited the eastern temperate forests of Canada and the northeastern US largely unsettled.

To help clarify this issue, we used mtDNA control region sequences [that have a diagnostic 3 bp insertion in gray wolves (Pilgrim et al. 1998)] from *Canis* samples (three individual teeth and one lower mandible with two in situ teeth) excavated from the pre-contact Lawson Iroquois Village (LIV) (c. 1530) located in London, Ontario, Canada (Fig. 2), along with morphometric data from the one mandible, to test the hypothesis that the wolf that occupied the region ~400 years before coyote expansion was a gray wolf.

Materials and methods

Sample collection and DNA extraction (Trent University Ancient DNA Lab)

Four *Canis* skull samples (LIVa3–LIVa6) were excavated from refuse-filled storage pits (middens) at the sixteenth century Lawson Iroquois Village site (AgHh-1) by researchers at the Museum of Ontario Archaeology (a research institute affiliated with the University of Western Ontario) in London, Ontario, Canada (Fig. 2). Recent radiocarbon analysis of carbonized corn dates the site and associated samples at 1530 A.D. Site description and archaeological significance are given in Warrick (2000). To increase the likelihood of sampling different individuals, we chose samples that were excavated from different middens. Three of the samples were individual teeth: two premolars (LIVa4, Museum Cat. No. AgHh-1 500-470 ss5 level 0-10 28614; LIVa5, Museum Cat. No. AgHh-1 4800535 3007 870412) and one canine (LIVa3, Museum Cat. No. AgHh-1 H8 15334 SN 583 584 585). The fourth was a 10 cm section of a lower mandible that had two in situ premolars (P3 and P4) (Fig. 3) (LIVa6, Museum Cat. No. AgHh-1 H8 15193 286), from which we removed the P4 premolar for DNA extraction.

Fig. 2 Location of the 2 hectare pre-contact Lawson Iroquois Village site (c. 1530 A.D.) (*star*) from where the four ancient *Canis* samples analysed in this study were excavated, and Algonquin Provincial Park (*dark gray polygon*) where eastern wolf skull samples (c. 1964/65) were collected. Eastern coyote skulls (c. 1974–1982) measured in this study were collected within ~100 km radius of the Lawson Iroquois Village site. Inset map is of eastern North America with Ontario *shaded in gray*

Fig. 3 Lower mandible with in situ premolars (P3 and P4) from *Canis* sample (LIVa6, Museum Cat. No. AgHh-1 H8 15193 286) excavated from the Lawson Iroquois Village in London, Ontario, Canada. This sample was used for both genetic and morphometric analyses

Ancient DNA studies are particularly susceptible to contamination (Willerslev and Cooper 2005) and sample degradation (Pääbo et al. 2004). They therefore require special precautions during extraction and amplification (Cooper and Poinar 2000), along with a critical analysis of results to assure authenticity (Gilbert et al. 2005). The following precautions were taken to minimize the risk of contamination and ensure reliability of results: samples were processed in a separate laboratory that is dedicated to the study of ancient DNA (free from contemporary samples and amplified product) where strict protocols that minimize the potential for contamination are enforced; the outside surface of each sample was scrubbed with a new toothbrush and DECON solution (1:49) then rinsed with DNAase-free ddH_20 (Gibco) prior to extraction; multiple negative controls were used during each step of the extraction and amplification to track potential contamination of reagents; and filter tips or disposable transfer pipettes were used throughout the process. In addition, multiple independent PCRs were conducted to confirm results.

Whole teeth were crushed with a hammer then placed in 3 ml 1× lysis buffer solution (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) and incubated overnight (approximately 18 h) at 37°C. We added 25 µl of Proteinase K (600 mAU/ml) (Qiagen, Mississauga) to each sample followed by incubation at 65°C for 1 h. Samples were then placed in a 65°C water bath and allowed to slowly cool inside a 37°C incubator at which time a second 25 µl volume of Proteinase K was added to each sample. All samples were then incubated for 6 days at 37°C and mixed by inversion every second day. DNA was extracted from the entire volume of solution with a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Mississauga) according to manufacturers directions, and eluted in 55 µl of 65°C AE buffer (Qiagen, Mississauga).

DNA amplification and sequence analysis

For each sample, the control region of the mitochondria was independently amplified six times in a 20 µl reaction

volume with 4 µl of DNA extract, 0.2 µM of each primer (Canid Primer F: 5′-GAA GCT CTT GCT CCA CCA TC-3′ (Pilgrim et al. 1998); Canid Primer R: 5′-GGG CCC GGA GCG AGA AGA GGG AC-3′ (Wilson et al. 2000)), 1× reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µg/µl bovine serum albumin (BSA), 1 unit of *Taq* polymerase (Invitrogen) and a total of 40 cycles. The region amplified is 343–347 base pairs in length and includes a diagnostic 3 base pair (bp) (GGT) insertion/deletion that distinguishes between New World (eastern wolf, red wolf, coyote) and Old World (gray wolf, dog) origin (Pilgrim et al. 1998). Sample LIVa5 was sequenced in both forward and reverse directions for three of the six independent PCRs. For the remaining samples, product from one PCR was re-amplified with the same primers, and the remaining 5 PCRs were re-amplified with a different reverse primer (CanCR3-R: 5′-GAG AAG AGG GAC ATT ACG AGC AAG-3′) whose sequence is located 11 bp within the initial amplified product and corresponds to positions 278–255 within the *Canis* mitochondrial D-loop (see Accession No. EU740415.1). Products were visualized with ethidium bromide on a 1.5% agarose gel, cleaned for sequencing with ExoSAP-IT (USB Corporation, Ohio) and analysed on a MegaBace 1000 with a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences, Baie d'Urfé, Quebec). All samples were sequenced multiple times. Amplified and/or re-amplified PCR product (from independent PCRs) was sequenced as follows: sample LIVa3 was sequenced 4 times (2× forward and 2× reverse), sample LIVa4 was sequenced with the forward primer 4 times and reverse primer 3 times; LIVa5 was sequenced both forward and reverse 3 times (with no re-amplification); and sample LIVa6 was sequenced 5 times in the forward direction and 4 times in the reverse direction. To resolve a 2 bp discrepancy in the direct sequences of sample LIVa6, we cloned fresh PCR product from that sample with a TOPO TA Cloning Kit (Invitrogen) according to manufacturer's directions and sequenced eight clones with both forward and reverse primers. Sequences were edited in 4Peaks v. 1.7 (Griekspoor and Groothuis 2006).

Independent replication (McMaster University Ancient DNA Centre)

Sample LIVa6 (section of lower mandible with in situ teeth) was sent to the McMaster University Ancient DNA Centre for independent replication. There was no sample remaining from the other three teeth, so independent replication was not possible for those samples. Sample preparation, DNA extraction, and PCR set-up took place in a facility specifically dedicated to the molecular evaluation of ancient material. Multiple extraction and amplification blanks were included throughout. A section of cortical bone was removed from the mandible, pulverized, and the 107 mg particulate was demineralized in 1.5 ml of 0.5 M EDTA, pH 8.0 under rotation at 25°C for 19 h. The sample was centrifuged and the supernatant (SN) purified with 500 µl PCI pH 8.0 (phenol/chloroform/isoamyl, 25:24:1), followed by 500 µl chloroform, and concentrated over Microcon YM-30 Centrifugal Filter Units (Millipore) with the filtrate collected after each filtration. Extracts were eluted in 100 µl $TE_{0.1}$ buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The bone pellet was further digested in 1.5 ml lysis buffer (10 mM Tris–HCl pH 8.0, 0.5% sarcosyl, 250 µg/ml protein kinase, 5 mM $CaCl_2$, 50 mM DTT, 1% PVP, and 2.5 mM PTB). The pellet and one extraction blank were rotated for 18 h at 55°C. Extracts were centrifuged and supernatants were purified via phenol/chloroform as described above.

Amplification was performed using the same PCR conditions used at Trent University, with one unit/µl of Ampli*Taq* Gold™ (Applied Biosystems) and 5 µl of extract per 20 µl reaction. PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions, and products were purified over 30 K PALL plates (Sigma–Aldrich) then eluted in 40 µl of $TE_{0.1}$. Sequencing of 10 clones was performed with ABI BigDye Terminator v. 1.1.

Phylogenetic analysis

Consensus sequences were aligned with Clustal in MEGA 4 (Tamura et al. 2007). Consensus sequences were compared to (1) previously published sequences acquired from GenBank (Vilà et al. 1997; Leonard et al. 2002), and (2) to 230 bp sequences from the *Canis* genetic database at the Natural Resources DNA Profiling and Forensic Centre (NRDPFC) at Trent University. Based on the results from ModelGenerator v. 0.85 (Keane et al. 2006), an HKY85 model of molecular evolution (Hasegawa et al. 1985) with gamma distributed rate variation was used to conduct phylogenetic analyses of these two datasets under a Bayesian statistical framework implemented in BEAST v. 1.4.7 (Drummond and Rambaut 2007). Consensus output and trees were formulated in LogCombiner v. 1.4.7 and TreeAnnotator v. 1.4.7 (modules within BEAST software), respectively from three independent runs of 10,000,000 generations with sampling from the chain every 1,000 steps. TRACER v. 1.4 (Rambaut and Drummond 2007) was used to assess the accuracy of the BEAST output, and FigTree (Rambaut 2008) was used to visualize consensus phylogenetic trees.

Morphometric analysis

Cranial measurements are often used as diagnostics that help determine North American species of *Canis* (Nowak

1979, 1995; Kolenosky and Standfield 1975; Schmitz and Kolenosky 1985). To further identify sample LIVa6 (that included part of the mandible as well as two in situ teeth Fig. 3) we compared morphometric data from this sample to modern day eastern wolves from Algonquin Provincial Park (sampled in 1964/65; $n = 30$; 11 females, 13 males, 6 unknown) and coyotes from southwestern Ontario (sampled between 1974 and 1982; $n = 36$; 14 females, 22 males). Three independent measurements of the following four variables from the lower mandible were taken with digital calipers: (1) maximum total length of P3 to P4 combined at enamel line, (2) width of mandible at the carnassial tooth, (3) maximum length of P4 at enamel line, and (4) maximum width of P4 at enamel line. Average measurements were used to construct box and whiskers plots for each dataset and measurements from sample LIVa6 were plotted independently to show group affinity.

Results

Sequencing and phylogenetic results

All four samples amplified in multiple independent PCRs and consensus sequences were determined. The number of sequences obtained for each sample and the length of the consensus sequences are as follows: LIVa3 = 4 (347 bp); LIVa4 = 7 (338 bp); LIVa5 = 6 (324 bp); LIVa6 = 9 (333 bp). For LIVa6, the consensus sequence determined from direct sequencing of the PCR product at Trent University had two nucleotide sites that could not be resolved between A/T (R) or G/C (Y). Five of the direct sequences were the same as the consensus sequence from cloning at Trent, but the subsequent four direct sequences had a 2 bp difference that matched sample LIVa4, suggesting either DNA damage (Hofreiter et al. 2001; Stiller et al. 2006) or some level of cross-contamination. (see Supplementary Fig. 1). Several of the cloned sequences from both Trent University and McMaster University had the same transitions (C → T, G → A) albeit at sporadic positions likely owing to damage in the template molecules, but the consensus sequence obtained by cloning at the two institutions was consistent. (Fig. 4, Supplementary Fig. 1), thereby resolving the inconsistencies in direct sequencing and alleviating concerns of possible cross-contamination.

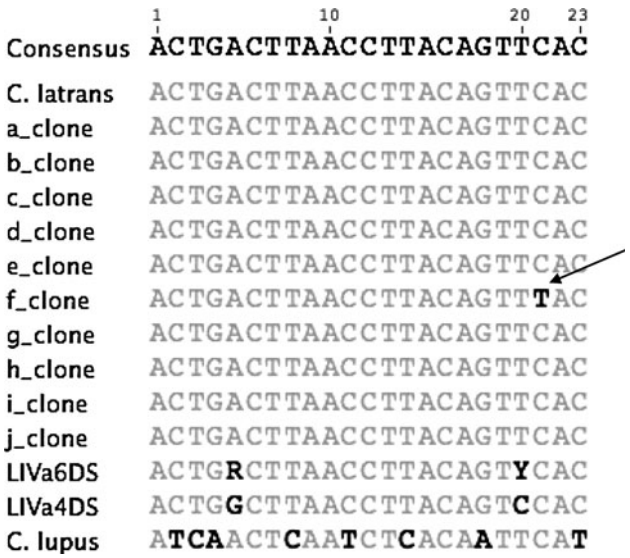

Fig. 4 Sequence alignment of 230 bp of the mitochondrial control region from 10 sequences of clones a–j (McMaster University) from LIVa6, the clones' Consensus sequence, two sequences from direct sequencing of LIVa6DS and LIVa4DS (Trent University), and 2 GENBANK sequences: one gray wolf (*C. lupus* DQ480508) and one coyote (C. *latrans* DQ480511). *R* and *T* indicate two sites that could not be resolved with direct sequencing but were later confirmed as A and T, respectively, by cloning at Trent University (Supplementary Fig. 1), thereby matching the consensus sequence found in clones from McMaster University. *Arrow* indicates at C → T transition resulting from DNA damage

None of the samples were of gray wolf origin, but two were of Old World origin and matched sequences previously found in ancient dog (*C. lupus familiaris*) samples: LIVa3 matches haplotype D3 (reported in Vilà et al. 1997; AF008146.1) found in 222–430 year old samples from Alaska (Leonard et al. 2002); and LIVa5 matches haplotype D28 reported in >1,000 year old samples from Bolivia (Leonard et al. 2002, Accession AY163882). The other two were New World sequences: the consensus sequence of LIVa6 was identical to that found in a western coyote (*C. latrans*) (Björnerfeldt et al. 2006, Accession DQ480511) and the consensus sequence for LIVa4 was 1 bp different from a western coyote sequence (Hailer and Leonard 2008, Accession FM209368.1). The phylogenetic tree clearly clusters samples LIVa3 and LIVa5 with ancient dog sequences within Clade I, and within that Clade, LIVa5 clusters within Clade *a*, a group of dog sequences that are unique to the New World (Leonard et al. 2002) (Fig. 5a). The two New World sequences cluster separately from both dog and gray wolf but group with western coyote sequences (Fig. 5a). When 230 bp were compared to eastern wolf and coyote sequences, the LIVa4 and LIVa6 cluster closely to sequences found in Saskatchewan coyotes (Fig. 5b).

Morphometric results

All four measurements of mandibles and teeth from present day eastern wolves ($n = 30$) and coyotes ($n = 36$) showed a distinct size difference between the two species, which is consistent with other data from cranial measurements (e.g., Nowak 1995). These results served as a frame of reference for categorizing the ancient New World mandible sample with in situ teeth (LIVa6), which was markedly larger than

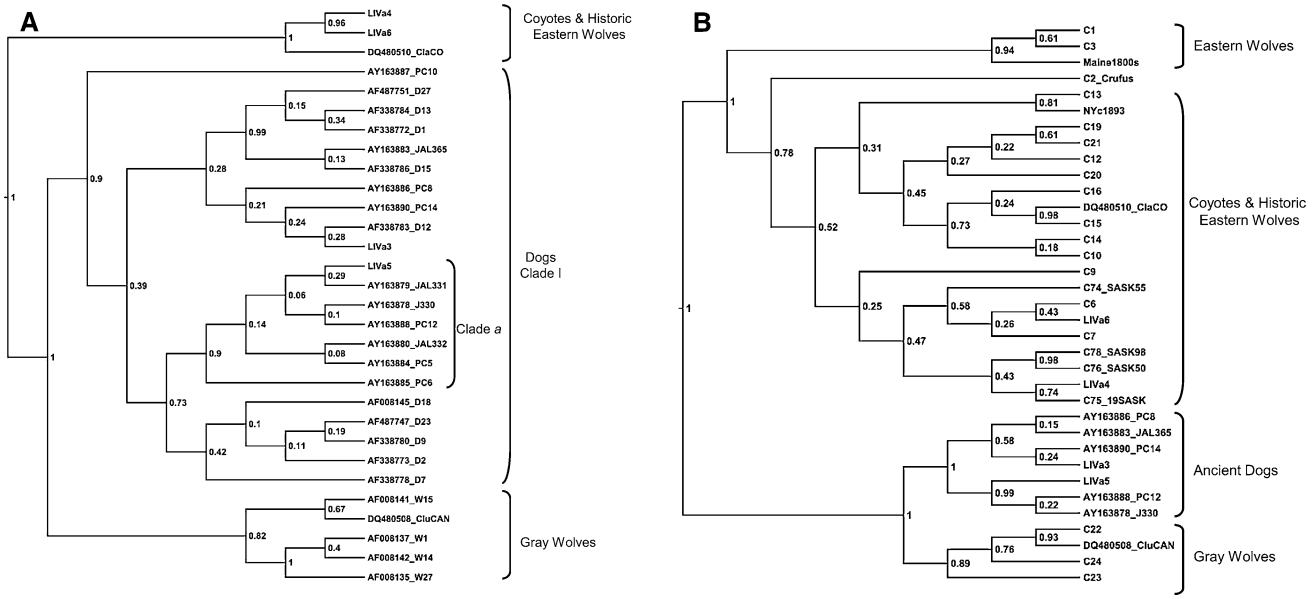

Fig. 5 **a** Cladogram (based on 308 bp) of gray wolf (*C. lupus*), dog (*C. lupus familiaris*), coyote (*C. latrans*) and ancient *Canis* mtDNA control region sequences from GENBANK (Accession numbers and haplotype names shown). Tree is based on Bayesian analysis in BEAST software. LIVa3–LIVa6 sequences are from *Canis* samples excavated from the Lawson Iroquois Village site in London, Ontario, Canada (c. 1530 A.D.). Node labels are Bayesian posterior probabilities. **b** Cladogram (based on 230 bp) of eastern wolf (*C. lycaon*) and coyote (*C. latrans*) mtDNA control region sequences from the *Canis* database at Trent University ("C" prefix) and ancient dog sequences from GENBANK (Accession numbers shown). *SASK* indicates sequences from samples collected in Saskatchewan, and ClaCO and CluCAN are from GENBANK (Accessions DQ480510 and DQ480508, respectively). Maine 1800s and NYc1893 are historic sequences from Wilson et al. (2003). C2_Crufus is the red wolf (*C. rufus*) sequence. Tree is based on Bayesian analysis in BEAST software. Samples LIVa4–LIVa6 are from *Canis* samples excavated from the Lawson Iroquois Village site in London, Ontario, Canada (c. 1530 A.D.). Node labels are Bayesian posterior probabilities

even the biggest eastern coyote sample and fell in the upper range of the eastern wolf samples (Fig. 6a–d). Based on these measurements, this sample of New World origin was grouped as an eastern wolf and not a coyote.

Discussion

The DNA sequence data allow us to reject the hypothesis that the ~500-year-old samples excavated from the Lawson Iroquois Village site were from "pure" gray wolves, and morphological measurements on the mandible sample with two in situ teeth, in conjunction with the historical record, rule out the possibility that the two samples with New World sequences were coyotes, despite having coyote-like mitochondrial haplotypes. Combined, these results suggest that the wolf that was historically present in the eastern temperate forests of southwestern Ontario was an eastern wolf (*C. lycaon*) that evolved in North America (New World), rather than a subspecies of gray wolf (*C. lupus*) that evolved in Eurasia (Old World). These results are consistent with the landscape and ecology of the area during the sixteenth century: the dense Carolinian forest and abundance of white-tailed deer (*Odocoileus virginianus*) (Pearce 1980), make it a much more suitable habitat for an eastern wolf than a coyote or a gray wolf. The data do not, however, reject an alternative hypothesis that the wolves could be of eastern-gray wolf hybrid origin as found in Minnesota (Wheeldon and White 2009).

The combined genetic and morphometric data clearly support previously published mitochondrial and nuclear data that suggest eastern wolves are distinct from gray wolves, and that they share a common evolutionary history in North America with coyotes (Wilson et al. 2000, 2003; Hedrick et al. 2002), independent of the gray wolf which evolved in Eurasia (Old World) and crossed the Bering land bridge into North America (New World) about 300,000 years ago (Vilà et al. 1997). Eastern wolves and western coyotes are morphologically distinct (Nowak 1979; Schmitz and Kolenosky 1985) and cranial measurements have been reliably used as a diagnostic to distinguish gray wolves, eastern wolves, and coyotes (Kolenosky and Standfield 1975; Nowak 1979, 1995; Schmitz and Kolenosky 1985).

Although a common evolutionary history may explain the shared mitochondrial haplotypes among eastern wolves and coyotes, our data are also consistent with previous mtDNA analysis of historic samples from New York and Maine that demonstrate the presence of "coyote" sequences in eastern wolves prior to coyote expansion (Wilson

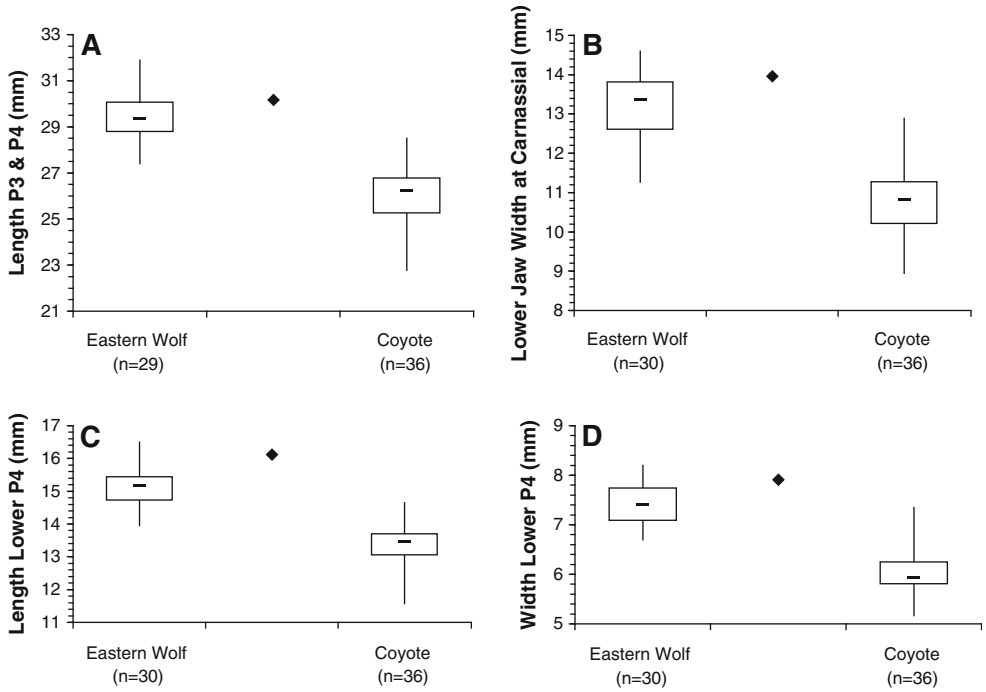

Fig. 6 **a–d** *Box* and *whiskers* plots comparing lower mandible and tooth measurements of eastern wolves (*C. lycaon*) from Algonquin Provincial Park and eastern coyotes (*C. latrans*) from southwestern Ontario. Diamonds show value for sample LIVa6. *Horizontal lines* within the *boxes* represent the median and *vertical lines* represent upper and lower quartiles

et al. 2003) suggesting that eastern wolves and coyotes may have come into contact and hybridized during the Wisconsin glaciation about 11,000–18,000 years ago. Additionally, recent genetic analysis of 100-year-old samples from the western Great Lakes region suggests that *C. lupus* × *C. lycaon* hybridization was already evident during a time when western coyotes were just beginning to colonize the area (Leonard and Wayne 2008; Wheeldon and White 2009; Koblmüller et al. 2009). Given the documented historic hybridization and the maternal inheritance of mitochondrial DNA, our results do not, therefore, reject the hypothesis that the animals present in southwestern Ontario 500 years ago were *C. lycaon* × *C. lupus* hybrids. Further data from nuclear or Y-chromosome markers are needed to test this hypothesis.

The presence of two ancient dog sequences, one found previously in samples from Bolivia and the other from Alaska, and the close clustering with samples from Latin America, suggest that dogs were widespread in the Americas prior to colonization in the mid sixteenth century. The clustering of sample LIVa5 with dog Clade *a* (within Clade I), a grouping that is confined to historic North American dogs and is not found in modern dogs, supports previous findings that indigenous dogs were largely replaced by those that accompanied European settlers during colonization (Leonard et al. 2002). However, the presence of a D3 haplotype from Clade I that is common in modern day dogs suggests that aboriginal dogs may have, to some extent, contributed genetic material to modern dog populations in North America.

Overall, these findings help to clarify the historic distribution of gray and eastern wolves in eastern North America. Although we recognize the small sample size and narrow distribution of our dataset limits broad scale conclusions regarding historic distribution of eastern wolves across eastern North America, our results add to the growing literature that suggests the southern limit of gray wolves did not likely include the eastern temperate forests of southwestern Ontario and the northeastern United States. In following reintroduction guidelines set by the International Union for Conservation of Nature (IUCN 1998), the data suggest that the eastern wolf (*C. lycaon*) is the most likely candidate species for reintroduction in northeastern US because there is little evidence that gray wolves historically occupied the eastern temperate forest ecological region. Analysis of more samples from a wider distribution, however, is needed to confirm this. However, any eastern wolf restoration efforts will be further confounded because "pure" populations of *C. lycaon* probably no longer exist. Even the population of eastern wolves in Algonquin Provincial Park in Ontario has been impacted by hybridization with coyotes and gray wolves (Grewal et al. 2004; Wilson et al. 2009).

While the ecosystem benefits of ensuring the persistence of a top predator are clear (Terborgh et al. 2001; Sergio et al. 2005; Chapron et al. 2008; Wallach et al. 2009), any

wolf restoration projects designed for northeastern North America also need to consider whether the larger eastern coyote, which contains eastern wolf genes through introgressive hybridization (Wilson et al. 2009), is starting to fill the ecological role of top predator. This question speaks to the broader ecological, ethical, philosophical, and political considerations of conservation science and its future direction (Schwartz and Vucetich 2009). Should we focus on preserving historical genetic states or conserving high levels of genetic diversity on which natural selection can act? In the absence of intervention, if eastern coyotes are able to effectively prey on large ungulates in areas where the eastern wolf was extirpated it may be that natural selection is in the process of compensating for the impacts of wolf extirpation and coyote colonization, thereby alleviating the ecological need for reintroduction efforts. If the goal is to conserve ecosystem integrity by re-establishing a wolf-like animal in eastern North America then conservation efforts that focus on protecting genetic variability and promoting gene flow among eastern *Canis* species via policies that provide widespread protection and promote reconnection of forested habitats is likely to be the most effective approach.

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References

Armstrong DP, Seddon PJ (2008) Directions in reintroduction biology. Trends Ecol Evol 23:20–25

Björnerfeldt S, Webster MT, Vilà C (2006) Relaxation of selective constraint on dog mitochondrial DNA following domestication. Genome Res 16:990–994

Chapron G, Andrén H, Liberg O (2008) Conserving top predators in ecosystems. Science 320:47

Cooper A, Poinar HN (2000) Ancient DNA: do it right or not at all. Science 289(5482):1139

Drummond AJ, Rambaut A (2007) Beast: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Frankham R, Ballou JD, Briscoe DA (2004) Introduction to conservation genetics. Cambridge University Press, Cambridge, UK

Gilbert MTP, Bandelt HJ, Hofreiter M, Barnes I (2005) Assessing ancient DNA studies. Trends Ecol Evol 20:541–544

Grewal SK, Wilson PJ, Kung TK, Shami K, Theberge MT, Theberge JB, White BN (2004) A genetic assessment of the eastern wolf (*Canis lycaon*) in Algonquin Provincial Park. J Mammal 85:625–632

Griekspoor A, Groothuis T (2006) 4Peaks http://mekentosj.com/4peaks/

Hailer F, Leonard JA (2008) Hybridization among three native North American *Canis* species in a region of natural sympatry. PLoS ONE 3:e3333

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174

Hedrick PW, Lee RN, Garrigan D (2002) Major histocompatibility complex variation in red wolves: evidence for common ancestry with coyotes and balancing selection. Mol Ecol 11:1905–1913

Hilton HH (1978) Systematics and ecology of the eastern coyote. In: Bekoff M (ed) Coyotes: biology, behavior and management. Academic Press, New York, pp 209–228

Hofreiter M, Jaenicke V, Serre D, von Haeseler A, Paabo S (2001) DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Res 29:4793

IUCN (World Conservation Union) (1998) Guidelines for reintroductions

Keane TM, Creevey CJ, Pentony MM, Naughton TJ, McLnerney JO (2006) Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6:29–45

Koblmüller S, Nord M, Wayne RK, Leonard JA (2009) Origin and status of the Great Lakes wolf. Mol Ecol 18:2313–2326. doi:10.1111/j.1365-294X.2009.04176.x

Kolenosky GB, Standfield OJ (1975) Morphological and ecological variation among gray wolves (*Canis lupus*) of Ontario, Canada. In: Fox MW (ed) The wild canids. Van Nostrand Reinhold, New York, pp 62–72

Kurtén B, Anderson E (1980) Pleistocene mammals of North America. Columbia University Press, New York

Kyle CJ, Johnson AR, Patterson BR, Wilson PJ, Shami K, Grewal SK, White BN (2006) Genetic nature of eastern wolves: past, present and future. Conservat Genet 7:273–287

Kyle CJ, Johnson AR, Patterson BR, Wilson PJ, White BN (2008) The conspecific nature of eastern and red wolves: conservation and management implications. Conserv Genet 9:699–701

Leonard JA (2008) Ancient DNA applications for wildlife conservation. Mol Ecol 17:4186–4196

Leonard JA, Wayne RK (2008) Native Great Lakes wolves were not restored. Biol Lett 4:95–98

Leonard JA, Wayne RK (2009) Invited reply. Wishful thinking: imagining that the current Great Lakes wolf is the same entity that existed historically. Biol Lett 5:67–68

Leonard JA, Wayne RK, Wheeler J, Valadez R, Guillen S, Vilà C (2002) Ancient DNA evidence for old world origin of new world dogs. Science 298:1613–1616

Mech LD (2009) Comment. Crying wolf: concluding that wolves were not restored. Biol Lett 5:65–66

Moore GC, Parker GR (1992) Colonization by the eastern coyote. In: Boer AH (ed) Ecology and management of the eastern Coyote. Wildlife Research Unit, University of New Brunswick, Fredericton, pp 23–38

Nowak RM (1979) North American quaternary *Canis*. University of Kansas Museum of Natural History Monograph No. 6

Nowak RM (1995) Another look at wolf taxonomy. In: Carbyn LN, Fritts SH, Seip DR (eds) Ecology and conservation of wolves in a changing world. Canadian Circumpolar Institute, University of Alberta, Edmonton, pp 375–398

Nowak RM (2002) The original status of wolves in eastern North America. SE Nat 1:95–130

Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645–679

Paquet PC, Strittholt JR, Staus NL (1999) Wolf reintroduction feasibility in the Adirondack Park. Conservation Biology Institute, Corvallis

Pearce RJ (1980) Lawson site (agHh-1) excavations, 1976–1979. Archaeological licence report submitted to the Ontario Ministry of Culture

Pilgrim KL, Boyd DK, Forbes SH (1998) Testing for wolf-coyote hybridization in the rocky mountains using mitochondrial DNA. J Wildl Manag 62:683–689

Rambaut A (2008) FigTree. http://tree.bio.ed.ac.uk/software/figtree/

Rambaut A, Drummond AJ (2007) Tracer v1.4. http://tree.bio.ed.ac.uk/software/tracer/

Sacks BN, Brown SK, Ernest HB (2004) Population structure of California coyotes corresponds to habitat-specific breaks and illuminates species history. Mol Ecol 13:1265–1275

Schmitz OJ, Kolenosky GB (1985) Wolves and coyotes in Ontario: morphological relationships and origins. Can J Zool 63:1130–1137

Schwartz MK, Vucetich JA (2009) Molecules and beyond: assessing the distinctness of the Great Lakes wolf. Mol Ecol 18:2307–2309

Seddon PJ, Armstrong DP, Maloney RF (2007) Developing the science of reintroduction biology. Conserv Biol 21:303–312

Sergio F, Newton I, Marchesi l (2005) Top predators and biodiversity. Nature 436:192

Smith DW, Peterson RO, Houston DB (2003) Yellowstone after wolves. Bioscience 53:330–340

Stiller M, Green RE, Ronan M, Simons JF, Du L, He W, Egholm M, Rothberg JM, Keates SG, Ovodov ND, Antipina EE, Baryshnikov GF, Kuzmin YV, Vasilevski AA, Wuenschell GE, Termini J, Hofreiter M, Jaenicke-Després V, Pääbo S (2006) Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. Proc Natl Acad Sci USA 103:13578–13584

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596

Terborgh J, Lopez L, Nunez P, Rao M, Shahabuddin G, Orihuela G, Riveros M, Ascanio R, Adler GH, Lambert TD, Balbas L (2001) Ecological meltdown in predator-free forest fragments. Science 294:1923–1926

Vilà C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, Honeycutt RL, Crandall KA, Lundeberg J, Wayne RK (1997) Multiple and ancient origins of the domestic dog. Science 276:1687–1689

Vonholdt BM, Stahler DR, Smith DW, Earl DA, Pollinger JP, Wayne RK (2008) The genealogy and genetic viability of reintroduced yellowstone grey wolves. Mol Ecol 17:252–274

Wallach AD, Murray BR, O'Neill AJ (2009) Can threatened species survive where the top predator is absent? Biol Conserv 142:43–52

Warrick G (2000) The precontact iroquoian occupation of southern Ontario. J World PreHistory 14:415–466

Wheeldon T, White BN (2009) Genetic analysis of historic western Great Lakes region wolf samples reveals early *Canis lupus/lycaon* hybridization. Biol Letters 5:101–104

Willerslev E, Cooper A (2005) Ancient DNA. Proc Biol Sci 272:3

Wilson PJ, Grewal S, Lawford ID, Heal JNM, Granacki AG, Penock D, Theberge JB, Theberge MT, Voigt DR, Waddell W, Chambers RE, Paquet PC, Goulet G, Cluff D, White BN (2000) DNA profiles of the eastern Canadian wolf and the red wolf provide evidence for a common evolutionary history independent of the gray wolf. Can J Zool 78:2156–2166

Wilson PJ, Grewal S, McFadden T, Chambers RC, White BN (2003) Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of gray wolf origin. Can J Zool 81:936–940

Wilson PJ, Grewal SK, Mallory FF, White BN (2009) Genetic characterization of hybrid wolves across Ontario. J Hered 100: S80–S89