

Considering Pleistocene North American wolves and coyotes in the eastern *Canis* origin story

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ABSTRACT

The evolutionary origins and hybridization patterns of *Canis* species in North America has been hotly debated for the past 30 years. Disentangling ancestry and timing of hybridization in Great Lakes wolves, eastern Canadian wolves, red wolves, and eastern coyotes is most often partitioned into a 2-species model that assigns all ancestry to grey wolves and/or coyotes, and a 3-species model that includes a third, North American evolved eastern wolf genome. The proposed models address recent or sometimes late Holocene hybridization events but have largely ignored Pleistocene era opportunities for hybridization that may have impacted the current mixed genomes in eastern Canada and the United States. Here, we re-analyze contemporary and ancient mitochondrial DNA genomes with Bayesian phylogenetic analyses to more accurately estimate divergence dates among lineages. We combine that with a review of the literature on Late Pleistocene *Canis* distributions to illuminate opportunities for ancient hybridization events between extinct Beringian grey wolves (*C. lupus*) and extinct large wolf-like coyotes (*C. latrans orcutti*) that we propose as a potentially unrecognized source of introgressed genomic variation within contemporary *Canis* genomes. These events speak to the potential origins of contemporary genomes and provide a new perspective on *Canis* ancestry, but do not influence/negate current conservation priorities of dwindling wolf populations with unique genomic signatures and key ecologically critical roles.

INTRODUCTION

Almost three decades ago, Wayne and Jenks (1991) proposed a gray wolf-coyote hybrid origin for the endangered red wolf (*Canis rufus*) in the United States. This conclusion, based on early genetic analysis with restriction enzymes and sequencing of the mitochondrial DNA (mtDNA) cytochrome b region, was met with harsh criticism from morphology experts who claimed the hybrid origin hypothesis was inconsistent with the fossil evidence and morphometric analysis of historical skull specimens (e.g. Nowak 1992). Nine years later, Wilson et al. (2000) used sequencing of the mtDNA control region in combination with microsatellite genotyping of wolves in Algonquin Park, Ontario to propose the “eastern wolf” (*C. lycaon*) as a North American evolved wolf, distinct from gray wolves (*C. lupus*) that originated in Eurasia, but closely related to coyotes (*C. latrans*) and red wolves that are endemic to North America. Since then, analyses of *Canis* evolutionary history have expanded to include genome-wide single-nucleotide polymorphism (SNP) markers and even whole genomes; researchers claim support for either a two-species model of *Canis* evolution in North America, whereby all ancestry can be attributed to gray wolves (*C. lupus*) or coyotes (*C. latrans*) (e.g. vonHoldt et al. 2011, 2016a) or a three-species model, wherein ancestry includes a third wolf-like species unique to eastern North America that encompasses both the eastern and red wolf (*C. lycaon/rufus*) (e.g. Rutledge et al. 2012a, 2015; Hohenlohe et al. 2017).

Despite general acceptance of a small eastern wolf with a predisposition for hybridizing with coyotes (Rutledge et al. 2010a, 2012b; Heppenheimer et al. 2018), there is additional debate on whether the red wolf is part of a larger eastern wolf lineage (assuming support for that model) (Wilson et al. 2000; Kyle et al. 2008; Rutledge et al. 2012a), or if eastern wolves represent coyote-introgressed red wolves that further hybridized with gray wolves at the northern edge of their historical range (Nowak 2002). Other *Canis* populations with contentious origins include: 1) the Great Lakes wolf (Leonard & Wayne, 2008; Koblmüller et al. 2009) that has been characterized as a gray wolf x coyote “eastern wolf” hybrid (*C. lupus x latrans*, vonHoldt et al. 2016a, 2016b), and alternatively as a gray

wolf x eastern wolf hybrid (*C. lupus x lycaon*, Wheeldon & White 2008; Mech 2011); and 2) the eastern coyote that has been described as a Great Lakes gray wolf x coyote hybrid (*C. lupus var. x C. latrans*, Kays et al. 2010) and an eastern wolf x coyote hybrid (*C. lycaon x latrans*; Wilson et al. 2009; Wheeldon et al. 2010; Wilson et al. 2012; Rutledge et al. 2010a, 2015).

For the most part, these debates focus on the contemporary hybridization between wolves and coyotes and how these interactions do or don't contribute to the origins of eastern North American *Canis*. This paradigm is likely an oversimplification of a complex system of *Canis* evolution. A number of studies have addressed the “enigmatic” nature of eastern wolves and the role of hybridization in their origin, with some explicitly testing or considering a three-species model (*C. lupus*, *C. latrans*, *C. lycaon/rufus*) (Hailer & Leonard 2008; Brzeski et al. 2016; Rutledge et al. 2010c, 2015; and see Hohenlohe et al. 2017; Heppenheimer et al. 2018a, 2018b, 2020). Ancestry is, however, frequently tested with the binary lineages of gray wolf and coyote without considering the potentially unique North American ancestry of *C. lycaon/rufus* (e.g. vonHoldt et al. 2011, 2016a, 2016b; Sinding et al. 2018). This omission may mask the contribution of this third lineage that is a sister species to coyotes.

Although analysis and genomic simulations of genome-wide SNPs provided support for the three-species model (Rutledge et al. 2015), these results could not resolve the possibility that eastern wolves arose from an ancient hybridization event followed by drift (Sefc and Koblmüller 2016; Rutledge et al. 2016). Typically, little consideration has been given to ancient hybridization models in the origins of eastern *Canis*, with most “ancient” DNA studies focused on early 20th century samples (Wilson et al. 2003; Koblmüller et al. 2009) or those from the very late Holocene (350 – 1900 years ago) (Rutledge et al. 2010b; Brzeski et al. 2016). These studies focus on modern forms of wolves and coyotes and not their pre-Holocene precursors, with some exception in considering the Beringian wolf (Leonard et al. 2007) or where the authors simply recognize the potential for ancient hybridization (Sefc & Koblmüller 2016; Rutledge et al 2015, 2016; Sinding et al. 2018). The vast majority of *Canis*

hybridization studies fail to consider the fossil-based morphological studies of Pleistocene and early Holocene *Canis* forms that acknowledge variable morphological characteristics, distributions, and demographic conditions that could facilitate and/or predispose ancient interactions that impact evolutionary processes (Nowak 1979; Nowak 2002; Meachen and Samuels 2012; Meachen et al. 2014; Meachen et al. 2016; Tomiya and Meachen 2018).

Although earlier analyses focused on mitochondrial DNA (e.g. Wilson et al. 2000, 2003; Leonard and Wayne 2008), microsatellite loci (e.g. Roy et al. 1994; Wilson et al. 2000; Wheeldon and White 2008), and Y-chromosomes (e.g. Fain et al. 2010, Wilson et al. 2012; Wheeldon et al. 2013), a growing number of studies have incorporated genome-wide SNPs to infer ancestry (Rutledge et al. 2015; vonHoldt et al. 2011, 2016a; Heppenheimer et al. 2018a; 2018b; 2020). However, the transition to genomic studies has done little to expand our understanding of the patterns of hybridization, with overall patterns from whole genomes reflecting similar patterns to early studies with several microsatellites. The utility of haploid markers for these purposes has recently been overshadowed by the allure of genome sequencing, but needs revisiting because genomic surveys have led to a projection of significantly shorter speciation timelines between the two most divergent *Canis* species, gray wolves (*C. lupus*) and coyotes (*C. latrans*). Specifically, vonHoldt et al. (2016a) proposed a 50 kya split based on whole genomes or even earlier based on mitogenomes (Schweizer and Wayne 2020), compared to 1-2 million years assumed for previous molecular dating studies using mitochondrial DNA (Lehman et al. 1991; Vilà et al. 1999; Wilson et al. 2000; Rutledge et al. 2010c) and originally based on fossil evidence (Nowak 1979). Clearly, divergence dating needs to be reconciled to more accurately delineate and reconstruct evolutionary lineages that will help characterize and resolve the origins of North American *Canis* species.

A review of previous and emerging literature reveals a significant range in the models of North American Pleistocene *Canis* evolution. First, there is potential for dispersal of gray wolves (*C. lupus*) from Beringian to more southern distributions inhabited by coyotes (*C. latrans*) and dire wolves (*C.*

dirus) prior to the end of the Last Glacial Maximum (LGM) and megafaunal extinctions approximately 13 kya (Heintzman et al. 2016) and 11 kya (Dundas 1999), respectively. Early pre-LGM *C. lupus* colonization of southern North America was originally proposed by Vilà et al. (1999) with additional supporting genetic (Koblmüller et al. 2016) as well as fossil evidence (Fig. 1) of Beringian wolves (Leonard et al. 2007) moving south prior to the LGM (Meachen et al. 2018). Recently, Loog et al. (2020) proposed that *C. lupus* populations only colonized North America from Beringia starting 15 kya years ago. Second, although there is a paucity of coyote genetic studies considering their Pleistocene history, fossil evidence supports the presence of a wolf-like coyote (*C. latrans orcutti*) prior to the Holocene (Nowak 1979; Meachen and Samuels 2012; Meachen et al. 2014; Tomiya and Meachen 2018). This “coyote” is an important consideration in evaluating the origins of the contemporary Great Lakes, eastern and red wolves as early contact and potential ancient hybridization would have likely consisted of the precursor Beringian *C. lupus*, proposed to be an extinct ecotype (Leonard et al. 2007), and the Pleistocene coyote, a wolf-like coyote that was larger than modern coyotes and emerged 10 kya (Meachen and Samuels 2012; Meachen et al. 2014; Tomiya and Meachen 2018).

In addition to the recent and extensive re-assessment of *Canis* fossil morphology, the presence of extensive mitochondrial datasets, including ancient *C. lupus*, provides an opportunity to more accurately calibrate the timing of species divergence. Accurate dating will allow more robust ancestral inference of critical haplotypes by addressing co-existence of forms and opportunity for ancient introgression events. Here, we re-evaluate the origins of contemporary *Canis* species within the framework of mtDNA divergence and from the perspective of late Pleistocene wolf and coyote distribution. We applied Bayesian approaches to previously published modern and ancient mtDNA datasets to calibrate substitution rates for estimating divergence times (Tong et al. 2018) between wolves and coyotes. We also used phylogenetic analyses to elucidate the presence of ancestral Pleistocene lineages within each species. Overall, we propose a new paradigm to test hypotheses of

Canis evolution that re-frames analyses with more accurate divergence times and in consideration of ancient Pleistocene types and their potential interactions.

MATERIALS & METHODS

We assessed Phylogenetic relationships and divergence times among mitogenome control region haplotypes using Bayesian methods. The software jModelTest 0.1.1 (Posada 2008) was applied to identify HKY+G as the best substitution model using the Bayesian Information Criterion for *Canis* control region haplotypes downloaded from GenBank (Supplemental S4). Two maximum clade credibility trees were created using BEAST v1.10.4 (Suchard et al. 2018) using time calibrated tips from ancient DNA derived haplotypes under a strict clock model, HKY+G substitution model, default optimization schedule, MCMC chain-length of 200 million, sampling every 20,000 generations and removing the first 10% of runs. The two independent runs were combined using the BEAST v1.10.4 package LogCombiner. We analyzed results from BEAST in Tracer v1.7 (Rambaut et al. 2018) and all effective sample sizes (ESS) were much greater than 200, indicating length of MCMC in accurately representing the posterior distribution was appropriate (Kuhner 2009). The phylogenetic trees we estimated were summarized in the BEAST v1.10.4 package TreeAnnotator and visualized in FigTree 1.4.4 (Rambaut 2016). Divergence times were calculated as the node heights of the 95% highest posterior density (HPD) intervals.

Modern and ancient whole mitochondrial DNA sequences were downloaded from GenBank (Supplemental S4) and aligned in Geneious R11.1.4 (Biomatters Ltd.) with ClustalW and default settings (UBC Cost matrix, Gap open cost: 15; Gap extend cost: 6.66). Alignment was trimmed on each end to have the same sequence length and annotated against the domestic dog mtDNA genome (CFU96639). We removed the control region to estimate divergence based on coding regions of the mtDNA genome. We used BEAST 2.6.0 (Bouckaert et al. 2019) to estimate divergence dates and create a phylogenetic tree based on modern and ancient samples. Partitions were assigned as in Loog et

al. (2020) with the following three independent mutation models: 1) PCDS1, rRNA, & tRNA with model HKY+I; 2) PCDS2 with model TrN+I; and 3) PCDS3 with model TrN+G. The positions of the partitions were identified based on start codons found from the reference genome annotations. The tree models for the partitions were linked and the site and clock models were unlinked. Substitution model parameters were set for each partition according to the recommended model. We used a strict clock and added tip dates for the ancient sequences based on the sample ages provided in the source reference. Parameterization of priors was set as described in Loog et al. (2020). Trees were sampled every 5,000 iterations over 50,000,000 iterations, with a burn-in of 10%. Tracer 1.7.1 was used to assure convergence of parameters and TreeAnnotator was used to determine the maximum clade credibility consensus tree. The final tree was visualized with FigTree 1.4.4.

We further estimated a minimum spanning tree haplotype network from mitogenome haplotypes using the randomized minimum spanning tree method in the R-package *pegus* (Paradis 2018); and generated a PHYML tree (Dereeper et al. 2008) using the mitochondrial control region sequences that considered insertion/deletions.

RESULTS & DISCUSSION

The Pleistocene North American landscape was inhabited by three wolf-like canids: the gray wolf (*C. lupus*); the coyote (*C. latrans*); and the dire wolf (*C. dirus*). Pleistocene coyotes and gray wolves have been characterized as morphologically different from their modern forms (Nowak 1979; Leonard et al. 2007; Meachen and Samuels 2012; Meachen et al. 2014; Tomiya and Meachen 2018), with no modern version of dire wolf due to its loss during the megafaunal extinctions (Dundas 1999). Incorporating Pleistocene forms of gray wolves and coyotes, their associated ancient lineages, and their potential interactions, has been limited in framing hypotheses and reconstructing the population histories of the eastern wolf, red wolf and Great Lakes wolf. Furthermore, estimates of mitochondrial DNA (mtDNA) divergence have assumed gray wolves and coyotes diverged 1-2 million years ago

based on the fossil evidence (Nowak 1979), an assumption that has carried over into the majority of molecular studies (e.g. Lehman et al. 1991; Vilà et al. 1999; Wilson et al. 2000; Rutledge et al. 2010c). As a result, a critical first test in reconstructing the population histories of North American *Canis* is calibrating the substitution rates and divergence times of regions of mtDNA with Bayesian derived phylogenies that include ancient haplotypes from fossils with reliable carbon-dating.

Bayesian reconstruction of two different mitochondrial DNA datasets, a 410 bp and 550 bp region of the control region, provided substitution rate estimates and the divergence times of a wolf-coyote split at approximately 7.38×10^{-7} and 4.12×10^{-7} , and 103 kya (74, 188 95% HPD) and 223 kya (90, 234 95% HPD), respectively. While significantly earlier than 1-2 million years ago, these are larger than divergence times based on whole nuclear genome analyses (vonHoldt et al. 2016) that dated the split to approximately 50 kya. A Bayesian phylogeny of whole mitochondrial DNA that included ancestral sequences and partitioned for different regions and 1st, 2nd and 3rd positions, derived 940 kya (737, 1,147 95% HPD) for the divergence of gray wolf and coyote. The discordant divergence times likely reflect the single gene regions used for control region versus the full mitogenomic sequences, the latter providing the more accurate dating estimates (Duchêne et al. 2011). This supports the proposed million-year gray wolf-coyote divergence assumption (e.g. Nowak 1979; Lehman et al. 1991, Wilson et al. 2000). While the smaller control region sequences had lower inter-specific timing estimates, they also had higher variance in the longer estimation than in more recently diverged intra-specific control region sequences.

Due to the absence of eastern and Great Lakes wolf full mitochondrial DNA sequences, we focused on two partial control region datasets: a 410 bp dataset (Fig. 1) that included ancient samples (Leonard et al. 2007), historical southern US wolf samples (Leonard et al. 2005) and representative eastern wolf/Great Lakes wolf haplotypes (Leonard & Wayne 2008; Kays et al. 2010), and a 550 bp dataset (Fig. 2) (Rashleigh et al. 2008; Fain et al. 2010; Thalmann et al. 2013; Ersmark et al. 2016) that was more limited in representative haplotypes but was assessed for concordance with the relatively

smaller control region segment. In general, similar topologies were observed between the two Bayesian analyses, specifically: ancestral positioning of Mexican, and southern wolf clades for the 410 bp reconstruction, in the *C. lupus* clade; and eastern/Great Lakes haplotypes as ancestral to the remaining *C. latrans* clade. Despite the similar topologies, the posteriors probabilities for the 550 bp analysis (Supplemental S2) were substantially more supportive than the 410 bp analysis (Supplemental S1). As a result, we applied a PhyML analysis (Fig. 3) and a Random Minimum Spanning Tree (RMSP) (Supplemental S3) to the 410 bp sequence set and confirmed the ancestral positioning of Mexican wolf/southern clade and eastern/Great Lakes wolf haplotypes to *C. lupus* and *C. latrans*, respectively.

As would be predicted, the sequences from ancient specimens (Leonard et al. 2007) were basal to modern gray wolf haplotypes, with one exception: the “southern” clade from early 1900s Mexican wolves (*C. lupus baileyi*) and the Plains wolf (*C. lupus nubilus*) that was flanked by now extinct Beringian wolf haplotypes (Fig. 1). The low posterior probability in these relationships limits the interpretation of this result, but the additional analyses presented here at a minimum supports the “southern” wolves as ancestral to other modern North American gray wolves. Beringian wolves as an ecotype of gray wolf have purportedly gone extinct (Leonard et al. 2007; Koblmüller et al. 2016), and although their corresponding ancient haplotypes are not seen in contemporary specimens, their role as progenitors to the southern modern wolves (*C. lupus baileyi* and *C. l. nubilus*) cannot be excluded. A similar basal position of the Mexican wolf was observed with the 550 bp sequence and has been consistently observed to be the most ancestral North American gray wolf (Vila et al. 1999; Thalmann et al. 2013; Sinding et al. 2018); its lineage originated approximately 25-30 kya (Fig 2) consistent with Koblmüller et al. (2016) prior to the closure of the Ice Free Corridor between the Cordilleran and Laurentide ice sheets during the Last Glacial Maxima (LGM) (23-13.5 kya; Heintzman et al. 2016). Interestingly, the 550 bp Bayesian phylogeny revealed modern *C. lupus* haplotypes flanked by ancient sequences from Ohio (Rashleigh et al. 2008), a region at the edge of the western range of Great Lakes, eastern and red wolves (Nowak 2002), and by contemporary coastal British Columbia sequences

(specifically Vancouver Island; Ersmark et al. 2016). These results further support pre-LGM southern dispersal or possibly a coastal refugial route 17-18 kya (Shafer et al. 2010; Darvill et al. 2018). The Ohio gray wolf sequences clustering with ancient sequences support the potential for very early contact between pre-LGM colonizing gray wolves and Pleistocene coyotes.

The role of the Beringian wolf in modern southern gray wolf (e.g. Mexican and Plains wolf; Leonard et al. 2005) evolution in North America is further supported by the recent fossil evidence from the Natural Pit site in Wyoming (Meachen et al. 2016). Evidence suggests these wolves colonized the south through the ice-free corridor dividing the Cordilleran and Laurentide ice sheets before the Last Glacial Maximum (LGM) and the maximal closure prior to 21,000 (Kleman et al. 2010) to 23,000 years ago (Heintzman et al. 2016). Multiple waves of gray wolf colonization in southern regions have been proposed, particularly in the evolution of the Mexican wolf (Vila et al. 1999; Thalmann et al. 2013). However, a single pre-LGM colonization event was interpreted from analysis of whole mtDNA genomes, with the proposal that modern gray wolves evolved south of the LGM and colonized north following the re-formation of the ice-free corridor to Beringia (Koblmüller et al. 2016). Recognition of the distinctiveness of the Mexican wolf was provided, with speculation that these wolves were structured from other evolving gray wolves south of the maximal ice sheets. In contrast, and based on a broader dataset, Loog et al. (2020) proposed that modern gray wolves colonized North America from Beringia starting 15 kya years ago. However, the basal ancestral position of Mexican wolves to other North American gray wolves, dating to the pre-LGM period of 30-35 kya in our analyses, was left as an open question in Loog et al. (2020) with the recognition of potential earlier colonization. Regardless of the role Beringia had as a cradle for modern *C. lupus* evolution, there is strong evidence of ancestral wolves south of the LGM that are likely candidates as being the progenitor of the southern wolf clade (Leonard et al. 2005). This association of the historical Mexican (*C. lupus baileyi*) and Plains wolf (*C. lupus nubilus*) clade with ancient Beringian wolf haplotypes (Leonard et al. 2007; Fig. 2) further supports the southern pre-LGM movement of Pleistocene gray wolves through an open glacial corridor

earlier than 23 kya. Interestingly, the distribution of FAUNMAP Rancholabrean (240 kya - 11 kya) fossils of gray wolf specimens (Fig. 4) prior to and into the LGM (Fig. 1), largely mapped to New Mexico and Wyoming, where the proposed corridor to the Natural Pit site is located (Meachen et al. 2016). This distribution pattern is concordant with the proposed distribution of the Mexican and Plains wolves (Nowak 2002; Leonard 2005).

Whereas fossil and genetic evidence support a southern presence of Pleistocene “Beringian” gray wolf, it is postulated that its distribution was constrained by the presence of the dire wolf (Meachen et al. 2012; Meachen et al 2016; Tomiya & Meachen 2018) until the megafaunal extinctions approximately 10 kya (Dundas 1999). Commonly sympatric with dire wolves was the Pleistocene coyote (*C. latrans orcutti*), a larger, more wolf-like canid than contemporary coyotes that only represent the most recent 10,000 years of the species evolutionary history. The coalescence of coyote-like mtDNA was approximately 30-60 kya in our Bayesian phylogenetic analyses of the control region, suggesting that the most ancestral lineages would correspond to the *C. latrans orcutti* subspecies that pre-date contemporary coyote lineages. Interestingly, the most basal *C. latrans* clade dating to the pre-LGM period were those sequences found in eastern wolves and Great Lakes wolves (Fig. 1). This well-defined clade supports an ancestral lineage to the eastern and Great Lakes wolves, and it does not exclusively validate the random introgression of modern coyote haplotypes within the range of natural variation of the species, although hybridization with modern coyotes and the *C. latrans* wolves (i.e. eastern wolves) has most likely occurred in both contemporary and historic times (Wilson et al. 2000, 2003, 2009). Of importance is that the ancestral eastern and Great Lakes wolf lineage in the context of purely *C. latrans* positioning (i.e. not factoring in ancient introgression) is on a time frame more conducive to sub-specific delineation than species-specific status given lineage dating in line with *C. latrans orcutti* and not 100-300 kya (Wilson et al. 2000).

Two extant Pleistocene lineages south of the LGM, one *C. lupus* and one *C. latrans*, raises the question whether these species had opportunity for ancient hybridization. Recent genomic

characterization has estimated the proportion of gray wolf and coyote admixture in the North American canids as gray wolf:coyote proportions of 70:30 for the Great Lakes wolf and eastern wolf combined, 30:70 for the red wolf, and 90:10 for the Mexican wolf (Sinding et al. 2018); similar proportions for one or more of these combinations have also been estimated elsewhere (vonHoldt et al. 2011; vonHoldt et al. 2016a; 2016b). This evidence supports introgressive hybridization but typically this is interpreted in the context of modern inter-breeding (with some exception see Sefc and Koblmüller 2015; Sinding et al. 2018). These studies applied the SABER analytical software to genome-wide SNP and whole genomes (vonHoldt et al. 2011; vonHoldt et al. 2016a). This approach, however, is limited in its ability to detect multiple hybridization events (e.g. past versus recent) (Supple and Shapiro 2018). Furthermore, the gradient of gray wolf-to-coyote ancestry may be expanded in that the pooling of eastern wolves, typically from Algonquin Provincial Park, with Great Lakes wolves may not be appropriate (see Rutledge et al. 2012a; Hohenlohe et al. 2017) as previous work shows them to have significantly less gray wolf genetic signal (Wilson et al. 2009; Rutledge et al. 2010a). Regardless, these findings support the opportunity for ancient hybridization between the Beringian wolf and the Pleistocene wolf-like coyote.

This hypothesis of Pleistocene hybridization is further supported by genetic evidence. A signature of potential ancestral mtDNA introgression may be associated with haplotype lu60, a coyote haplotype found in the Mexican wolf (Leonard et al. 2005). This lineage extends into the Pleistocene (18 kya (Fig. 2)), a time that pre-dates modern coyotes, when *C. latrans orcutti* inhabited the landscape. The lu60 haplotype is related to a single observed coyote sequence (la86) found only in Texas, a geographic region that overlaps part of the historical range of the Mexican wolf (Hendricks et al. 2016). The absence of lu60 and highly similar sequences in modern coyotes further supports a more ancient event, particularly given the maintenance of a high contemporary haplotypic diversity in extant coyotes. Furthermore, surveys of interspecific gene flow among *Canis* identified support for ancient hybridization, including: 1) introgression from the Mexican wolf lineage into coyotes (Gopalakrishnan

et al. 2018) and *vice versa* (Sinding et al. 2018); 2) the generation of novel population-specific alleles in eastern wolves (vonHoldt et al. 2016a; Sinding et al. 2018) including differentiation between Great Lakes and eastern wolves (Sinding et al. 2018); and 3) relatively consistent levels of wolf versus coyote genetic makeup in Great Lakes and eastern wolves (Sinding et al. 2018) supporting a more historical introgression event. Although this evidence does not reject the more recent hybridization that has clearly taken place (e.g. Wilson et al. 2000, 2003), these contemporary signatures also support our proposed ancient hybridization between Pleistocene coyotes and Beringian wolves that could have contributed to modern introgressive signatures.

Resolving the hypothesis of ancient hybridization between Beringian wolves and Pleistocene coyotes and the impact on the ancestry of contemporary North American *Canis* requires genetic and morphometric data from additional ancient specimens (e.g. Beringian wolf skulls and *C. latrans orcutti*, respectively, from the Wyoming Natural Trap site (Meachen et al. 2016)). Although evidence supports the pre-LGM southward movement of Beringian wolves, there is also some evidence to suggest opportunities for northward movement of *C. latrans orcutti*. More specifically, a 47 kya fossil from the Yukon has been identified morphologically as a “coyote” (Fig. 4). Expansion on the existing ancient DNA dataset associated with Beringian wolves (Leonard et al. 2007) by obtaining more specimens and/or expanding into genomic-based markers would further refine the evolutionary story and relationship of Pleistocene *Canis* species. Overall, the distribution of Pleistocene wolves and coyotes south of the Collideran/Laurentide ice sheets during the LGM, and signatures of older introgression, support the likelihood that ancient hybridization has shaped the ancestry of extant wolves and coyotes in eastern regions of North America, where contemporary hybridization patterns have muddled ancestry patterns based on nuclear genome scans.

These inferences provide a new perspective that could re-shape our understanding of North American *Canis* ancestral origins. Re-calibrating the gray wolf-coyote divergence time allows for more accurate estimates of deep ancestry and supports eastern wolves as ancestral to modern *C. latrans*. This

suggested nomenclature is based purely on the timing of divergence and not on the ecological adaptation and/or speciation of modern eastern wolves and coyotes. Given the different niches occupied by eastern wolves and coyotes, along with the potential association of modern eastern wolves with the more wolf-like *C. latrans orcutti*, we suggest the common name “wolf” is most appropriate for eastern wolves, regardless of admixture with ancient Beringian wolves or modern gray wolves. The taxonomic nomenclature, given differential hybridization with gray wolves and coyotes, depending on the eastern wolf in question, is significantly more complex, and more targeted research will be required to move beyond the binary modern gray wolf x coyote hybridization commonly utilized (vonHoldt et al. 2011; vonHoldt et al. 2016a; Sinding et al. 2018) versus distinct species lineage (Wilson et al. 2000; Rutledge et al. 2015). Furthermore, future research should include contributions from *C. dirus* into the contemporary *Canis* genome complex.

By combining a review of recent fossil evidence and Pleistocene *Canis* distributions with a re-analysis of existing ancient and modern mitochondrial DNA data, we have introduced a more comprehensive evolutionary framework based on potential ancient interactions by which to test hypotheses of North American *Canis* ancestry. Based on our assessment, future research should consider several specific aspects to improve our understanding of *Canis* species origins:

1. Gray wolves and coyotes diverged close to 1 million years ago based on mitochondrial DNA divergence consistent with previously proposed timing;
2. Gray wolves appear to have colonized the southern distribution of the United States prior to the LGM before the ice sheets closed from 23 to 13 kya;
3. The Beringian wolf may well have been the ecotype that was the progenitor to the southern wolf clade of the Mexican wolf (*C. lupus baileyi*) and potentially the Plains wolf (*C. lupus nubilus*);
4. The “coyote” on the landscape during the Beringian wolf southern colonization until the end of the Pleistocene was a larger more wolf-like animal;

5. Ancient hybridization, i.e. prior to the Holocene (11kya), may have involved the Beringian wolf and the large wolf-like Pleistocene coyote. Interbreeding seems possible given the wolf-like nature of both forms compared to today's more divergent morphological forms where natural viable gray wolf x coyote hybridization in western regions is absent;
6. North American *Canis* operate along a range of hybrid ancestries potentially contributed to by contemporary and ancient interbreeding;
7. The formal naming of the wolves in eastern North America will be dependent on whether ancient hybridization has contributed to a formal eastern wolf species, e.g. *C. lycaon*, with its range of contemporary hybrids, or a subspecific assignment requires consideration of *C. latrans orcutti* within taxonomic designations (Table 1) reflective of Pleistocene events and the modern eastern wolves.

Although this current study does not resolve the question of the number and nomenclature of eastern North American wolf species, it is nonetheless an important step to refocus a decades long unresolved debate on the evolution of North American wolves.

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FIGURE LEGENDS

Figure 1. Dated BEAST phylogeny of 405 bp control region. Green branches are ancient Beringian wolf haplotypes, Blue are Mexican wolf and southern clade haplotypes, and red are Great Lakes and eastern wolf haplotypes. The gray area represents the estimated timing the Cordilleran and Laurentide Ice Sheets closed any corridor from Beringia to North America south of the ice sheet 22,000 to 13,000 years ago.

Figure 2. Dated BEAST phylogeny of 550 bp control region. Great Lakes and eastern wolf haplotypes are represented by Cly1 and Cly2 (Ersmark et al. 2016). The gray area represents the estimated timing the Cordilleran and Laurentide Ice Sheets closed any corridor from Beringia to North America south of the ice sheet 22,000 to 13,000 years ago.

Figure 3. PhyML tree plotted with GrapeTree of 410 bp control region. Green are ancient Beringian wolves, Blue are Mexican wolves, and red are Great Lakes and eastern wolves. Posterior probabilities are presented for critical nodes.

Figure 4. Distribution of *Canis latrans* (A) and *lupus* (B) Faunmap fossils through the Rancholabrean with maximum ice sheets estimated at 30,000 years ago (ya) (Batchelor et al. 2019). **A)** Distribution of *Canis latrans* fossils by province/state and minimum and maximum age estimates: **1) YU:** 47,170-47,170 ya; **2) AB:** 36,800-39,000 ya; **3) OR:** 35,000-65,000 ya; **4) ID:** 21,000-33,000 ya, 21,000-33,000 ya, 75,000-125,000 ya, 15,000-72,000 ya, 58,000-86,000 ya; **5) CA:** 23,000-27,000 ya, 27,000-34,000 ya, 26,000-32,000 ya, 30,000-35,000 ya, 67,000-112,000 ya, 40,000-110,000 ya; **6) NV:** 40,000-110,000 ya; **7) UT:** 40,000-100,000 ya; **8) AZ:** 31,000-110,000 ya; **9) NM:** 13,000-25,000 ya, 13,500-20,000 ya, 25,000-35,000 ya, 25,000-35,000ya, 20,120-25,000 ya; **10) SD:** 26,075-26,075 ya; **11) TX:** 25,000-35,000 ya, 31,400-35,000 ya, 23,230-23,230 ya; **12) IN:** 24,390-25,710 ya; **13) WV:** 17,060-29,400; and **14) PA:** 13,740-13,740 ya; 11,000-11,000 ya. **B)** Distribution of *Canis lupus* fossils by province/state and minimum and maximum age estimates: **1) YU:** 20,780-49,400 ya, 30,500-34,000 ya; 27,270-28,570 ya; **2) AB:** 25,960-44,800 ya; **3) OR:** 35,000-65,000 ya; **4) WY:** 12,777-15,500 ya, 15,500-20,250 ya, 13,500-27,000; **5) SD:** 26,075-26,075 ya; **6) UT:** 14,500-18,000 ya; **7) AZ:** 31,000-110,000 ya; **8) NM:** 13,000-25,000 ya, 13,500-20,000 ya, 15,030-30,000 ya, 25,000-35,000 ya, 25,000-35,000ya, 20,120-25,000 ya; and **9) GA:** 13,560-24,080 ya.

DATA ACCESSIBILITY STATEMENT

All sequence data are available through the Genbank public data repository. Genbank Accessions for DNA sequences used in the control region and whole mtDNA genome analysis can be found in the Supplemental S4.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Paul A. Wilson: Conceptualization (lead); Data curation (equal); Formal analysis (lead); Investigation (lead); Methodology (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal). **Linda Y. Rutledge:** Conceptualization (supporting); Data curation (equal); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Visualization (supporting); Writing-original draft (supporting); Writing-review & editing (equal).

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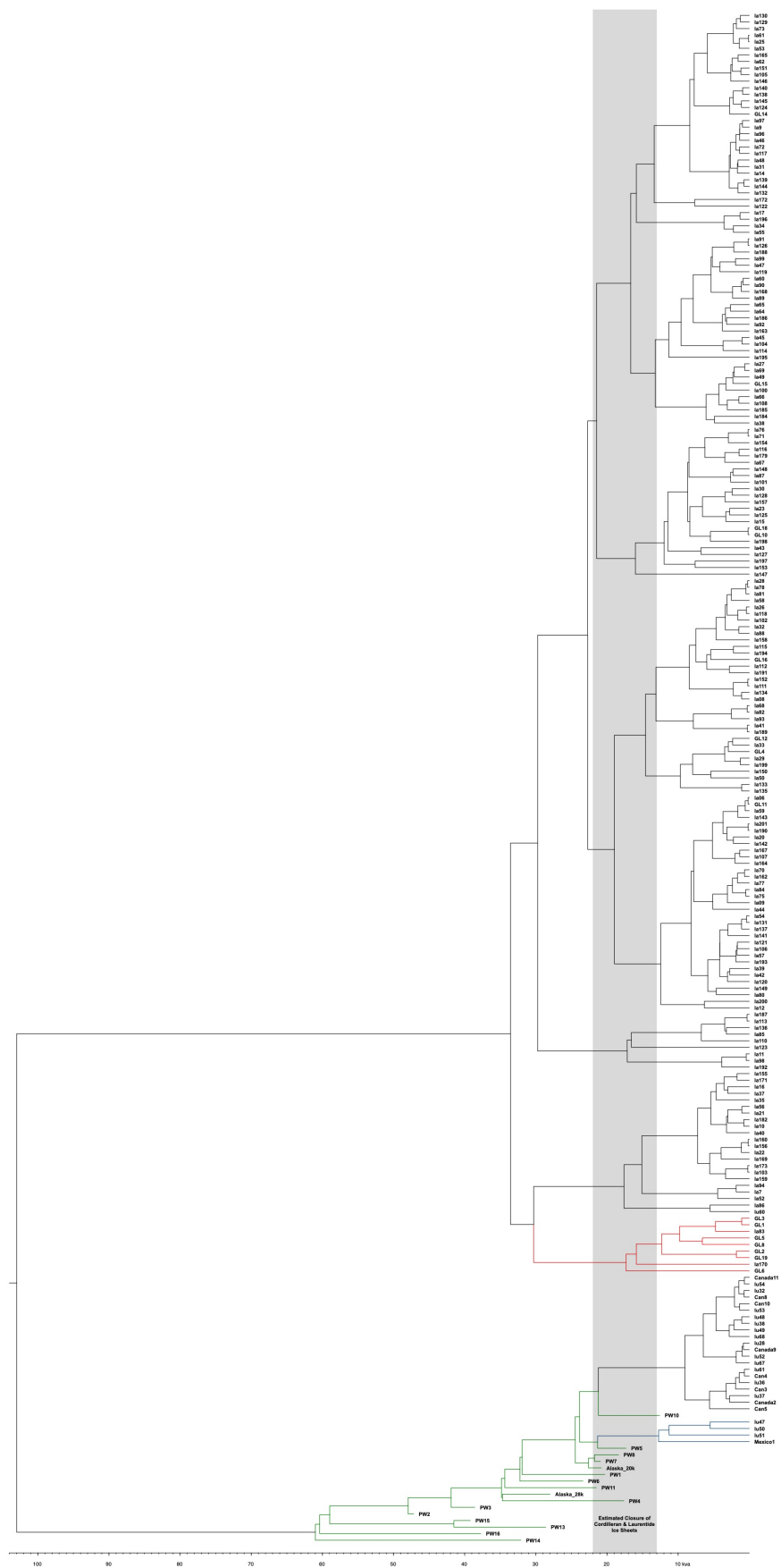


Figure 1

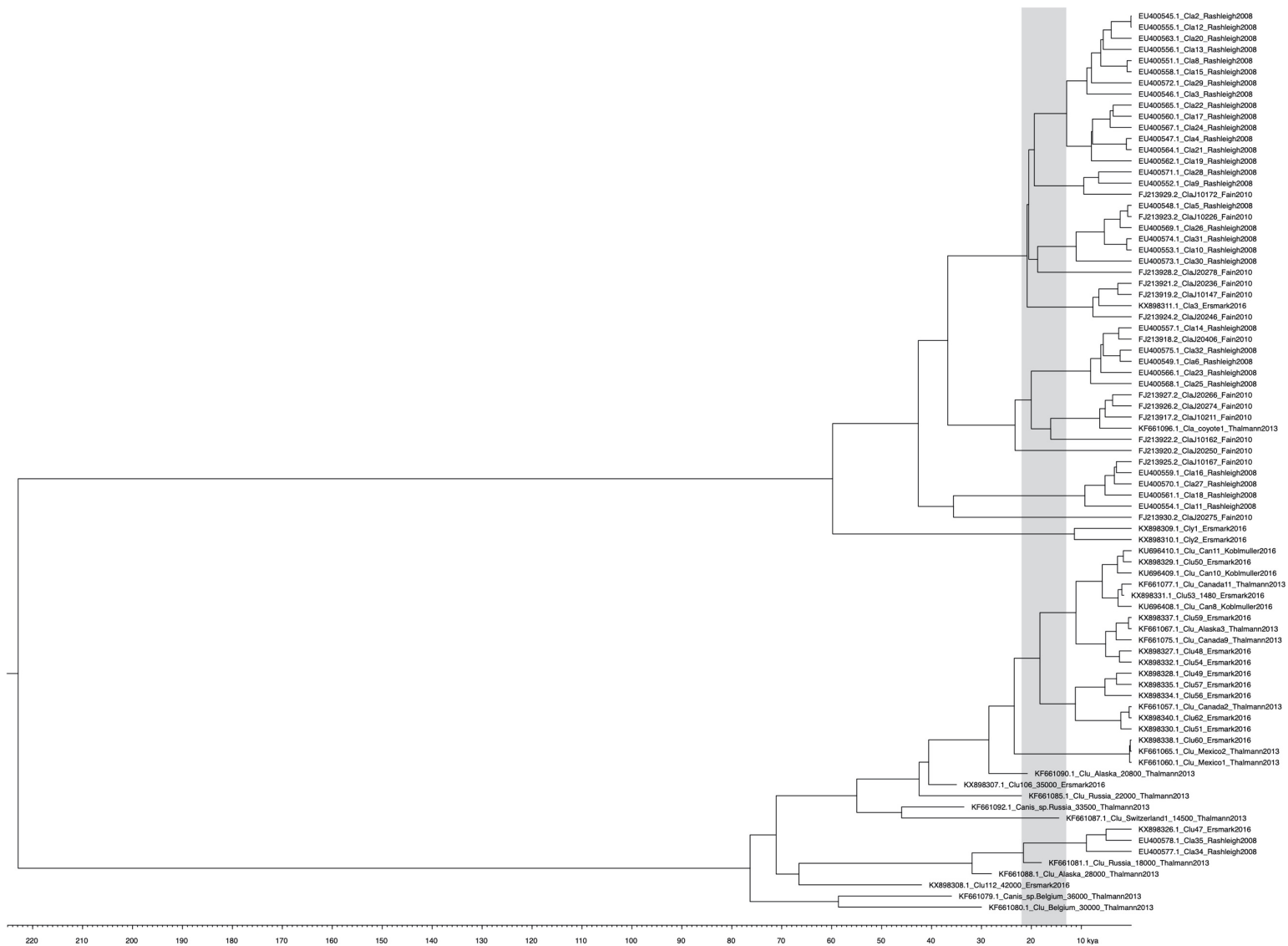


Figure 2



Figure 3



Figure 4A



Figure 4B

SUPPLEMENTARY MATERIAL

Figure S1. Bayesian maximum clade credibility tree of 405 bp region of the mitochondrial DNA control region showing nodal support ($\geq 60\%$) and node bars (blue) indicating the 95% confidence intervals for the age of nodes. Time is presented in thousands of years ago (kya). Sample codes from published sources with the following GenBank Accession codes: AY codes (Leonard et al. 2005); FM “lu” codes (Munoz-Fuentes et al. 2009); FM “la” codes (Hailer & Leonard 2008); GQ “GL” codes (Leonard & Wayne 2008); GQ “la”, “lu” codes (Koblmüller et al. 2009); GQ “cla” codes (Kays et al. 2010); JN “la” codes (Koblmüller et al. 2012); KF codes (Thalmann et al. 2013); KU codes (Koblmüller et al. 2016); and PW codes with age of sample in years (ya) (Leonard et al. 2005). Unique and identical haplotypes are grouped in parentheses [: [AY812730.1 lu37, KU696411.1 Ala1]; [AY812732.1 lu47]; [AY812733.1 lu48]; [AY812734.1 lu49]; [AY812735.1 lu50]; [AY812736.1 lu51]; [AY812737.1 lu52]; [AY812738.1 lu53]; [AY812739.1 lu54]; [AY812740.1 lu60]; [AY812741.1 lu61]; [FM201608.1 lu28, KU696400.1 Can1, KU696406.1 Can7]; [FM201609.1 lu68]; [FM201631.1 lu36, KF661066.1 Alaska2]; [FM201633.1 lu29, KF661059.1 Canada4, KF661071.1 Alaska4, KF661073.1 Alaska6, KU696404.1 Can5]; [FM201641.1 lu31, KF661064.1 USA1, KF661068.1 USA2, KF661069.1 USA3, KF661072.1 Alaska5K, U696403.1 Can4]; [FM201672.1 lu67]; [FM201774.1 lu30, KF661074.1 Canada8, KU696402.1 Can3]; [FM209365.1 la06]; [FM209366.1 la08]; [FM209367.1 la11]; [FM209368.1 la27]; [FM209369.1 la35]; [FM209370.1 la54]; [FM209371.1 la86]; [FM209372.1 la87]; [FM209373.1 la111]; [FM209374.1 la131]; [FM209375.1 la132]; [FM209376.1 la133]; [FM209377.1 la134]; [FM209378.1 la135]; [FM209379.1 la136]; [FM209380.1 la137]; [FM209381.1 la138]; [FM209382.1 la139]; [FM209383.1 la140]; [FM209384.1 la141]; [FM209385.1 la142]; [FM209386.1 la143]; [FM209387.1 la144]; [FM209388.1 la145]; [FM209389.1 la146]; [FM209390.1 la147]; [FM209391.1 la12]; [FM209392.1 la17]; [FM209393.1 la21]; [FM209394.1 la23]; [FM209395.1 la25, FM209419.1 la74]; [FM209396.1 la26]; [FM209397.1 la28]; [FM209398.1 la30]; [FM209399.1 la31]; [FM209400.1 la32]; [FM209401.1 la33]; [FM209402.1 la34]; [FM209403.1 la36]; [FM209404.1 la37]; [FM209405.1 la38]; [FM209406.1 la39]; [FM209407.1 la40]; [FM209408.1 la41, FM209417.1 la51, JN982568.1 la41]; [FM209409.1 la42]; [FM209410.1 la44]; [FM209411.1 la45]; [FM209412.1 la46]; [FM209413.1 la47]; [FM209414.1 la48]; [FM209415.1 la49]; [FM209416.1 la50]; [FM209418.1 la52]; [FM209420.1 la75]; [FM209421.1 la76]; [FM209422.1 la123]; [FM209423.1 la125]; [FM209424.1 la127]; [FM209425.1 la128]; [GQ849342.1 GL1, GQ863717.1 GL20]; [GQ849351.1 GL2]; [GQ849352.1 GL3]; [GQ849353.1 GL4]; [GQ849354.1 GL5]; [GQ849355.1 GL6]; [GQ849357.1 GL8]; [GQ849359.1 GL10, GQ849366.1 GL17, GQ849369.1 Cly voucher GL10]; [GQ849360.1 GL11]; [GQ849361.1 GL12]; [GQ849362.1 GL13]; [GQ849363.1 GL14, JN982590.1 la79]; [GQ849364.1 GL15]; [GQ849365.1 GL16, GQ849372.1 la18]; [GQ849367.1 GL18]; [GQ849368.1 GL19]; [GQ849370.1 lu32, KU696407.1 Can9, KU696401.1 Can2]; [GQ849371.1 la15]; [GQ849373.1 la19]; [GQ849374.1 la20]; [GQ849375.1 la29]; [GQ849376.1 la64]; [GQ849377.1 la65]; [GQ849378.1 la91]; [GQ849380.1 la97]; [GQ849381.1 la98]; [GQ849382.1 la99]; [GQ849383.1 la107]; [GQ849384.1 la108]; [GQ849385.1 la121]; [GQ849386.1 la124]; [GQ849387.1 la148]; [GQ849388.1 la149]; [GQ849389.1 la150]; [GQ849390.1 la151]; [GQ849391.1 la152]; [GQ863726.1 cla35]; [GQ863728.1 cla37]; [GQ863732.1 cla41]; [JN982562.1 la7]; [JN982563.1 la9]; [JN982564.1 la10]; [JN982565.1 la14]; [JN982566.1 la16]; [JN982567.1 la22]; [JN982569.1 la43]; [JN982570.1 la53]; [JN982571.1 la55]; [JN982572.1 la56]; [JN982573.1 la57]; [JN982574.1 la58]; [JN982575.1 la59]; [JN982576.1 la60]; [JN982577.1 la61]; [JN982578.1 la62]; [JN982580.1 la66]; [JN982581.1 la67]; [JN982582.1 la68]; [JN982583.1 la69]; [JN982584.1 la70]; [JN982585.1 la71]; [JN982586.1 la72]; [JN982587.1 la73]; [JN982588.1 la77]; [JN982589.1 la78]; [JN982591.1 la80]; [JN982592.1 la81]; [JN982593.1 la82]; [JN982594.1 la83]; [JN982595.1 la84]; [JN982596.1 la85]; [JN982597.1 la88]; [JN982598.1 la89]; [JN982599.1 la90]; [JN982600.1 la92]; [JN982601.1 la93]; [JN982602.1

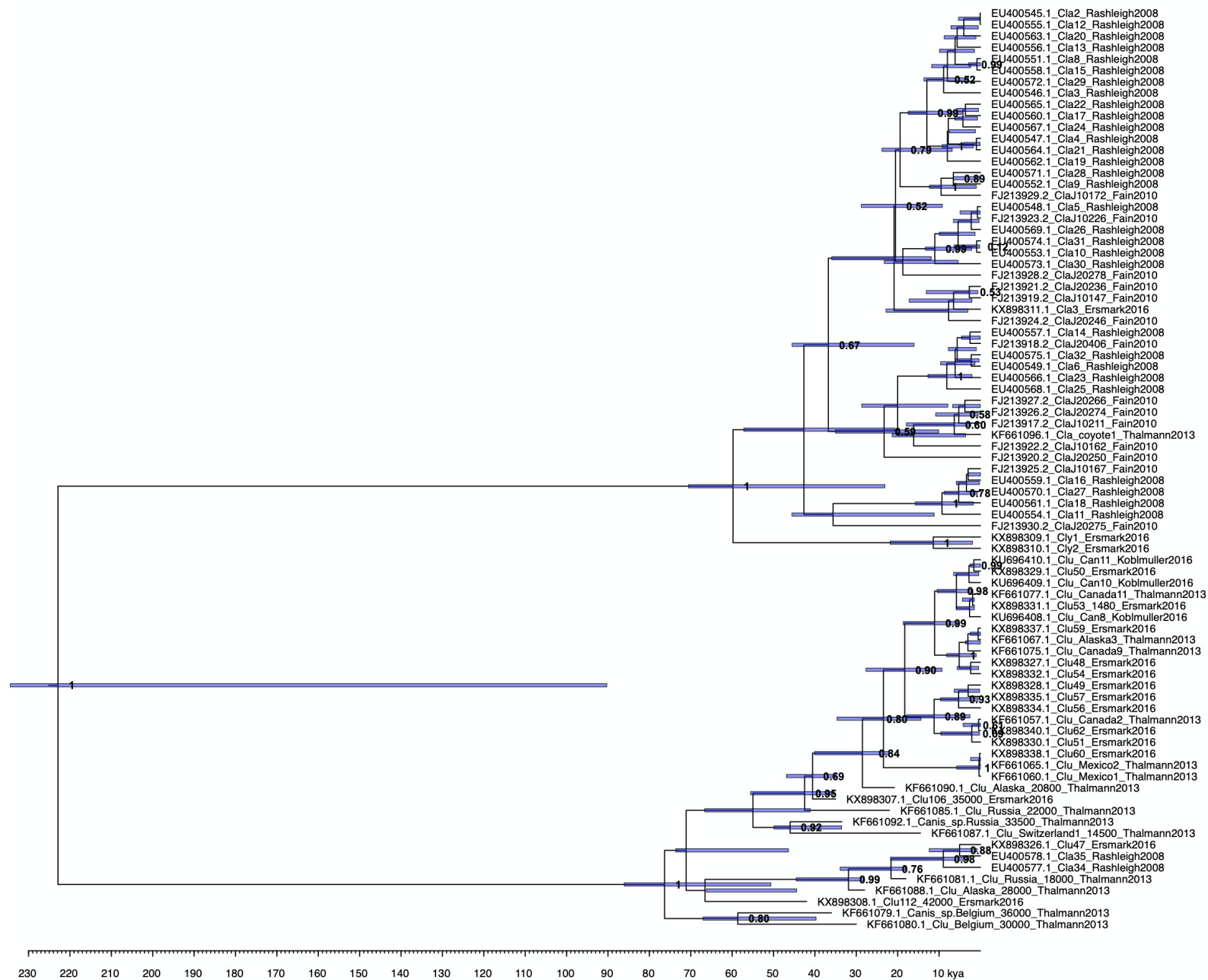
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Figure S2. Bayesian maximum clade credibility tree of 550 bp region of the mitochondrial DNA control region showing nodal support ($\geq 60\%$) and node bars (blue) indicating the 95% confidence intervals for the age of nodes. Time is presented in thousands of years ago (kya). Sample codes from published sources with the following GenBank Accession codes: EU codes (Rashleigh et al. 2005); FJ codes (Fain et al. 2010); KX codes (Ersmark et al. 2016); KF codes with age of sample in years (ya) (Thalmann et al 2013); KU codes (Koblmüller et al. 2016); Unique and identical haplotypes are grouped in parentheses []: [EU400554.1 Cla11]; [EU400561.1 Cla18]; [EU400570.1 Cla27]; [EU400559.1 Cla16]; [FJ213925.2 ClaJ10167]; [KX898310.1 Cly2, FJ213914.2 CluE40920]; [KX898309.1 Cly1, FJ213916.2 CluD21046]; [FJ213930.2 ClaJ20275]; [FJ213928.2 ClaJ20278]; [FJ213920.2 ClaJ20250, EU400576.1 Cla33]; [EU400568.1 Cla25]; [EU400557.1 Cla14]; [EU400549.1 Cla6]; [EU400566.1 Cla23]; [EU400575.1 Cla32]; [FJ213918.2 ClaJ20406, EU400544.1 Cla1]; [EU400560.1 Cla17]; [EU400562.1 Cla19]; [EU400564.1 Cla21]; [EU400565.1 Cla22]; [EU400567.1 Cla24]; [EU400547.1 Cla4]; [EU400552.1 Cla9]; [EU400571.1 Cla28]; [FJ213929.2 ClaJ10172]; [EU400572.1 Cla29]; [EU400558.1 Cla15]; [EU400551.1 Cla8]; [EU400546.1 Cla3]; [EU400555.1 Cla12]; [EU400556.1 Cla13]; [EU400563.1 Cla20]; [EU400545.1 Cla2]; [KF661096.1 Cla coyote1]; [EU400573.1 Cla30]; [EU400574.1 Cla31]; [FJ213923.2 ClaJ10226, EU400550.1 Cla7]; [EU400553.1 Cla10]; [EU400569.1 Cla26]; [EU400548.1 Cla5]; [FJ213922.2 ClaJ10162]; [FJ213926.2 ClaJ20274]; [FJ213917.2 ClaJ10211]; [FJ213927.2 ClaJ20266]; [FJ213919.2 ClaJ10147]; [KX898311.1 Cla3, FJ213915.2 CluH11321]; [FJ213921.2 ClaJ20236]; [FJ213924.2 ClaJ20246]; [KF661079.1 Canis sp.Belgium 36,000 ya]; [KF661085.1 Clu Russia 22,000 ya]; [KF661065.1 Clu Mexico2]; [KX898338.1 Clu60]; [KF661060.1 Clu Mexico1]; [KX898332.1 Clu54]; [KF661075.1 Clu Canada9]; [KU696406.1 Clu Can7, KU696400.1 Clu Can1, FJ213913.2 CluE11254, KX898337.1 Clu59]; [KF661058.1 Clu Alaska1, KF661067.1 Clu Alaska3]; [KX898327.1 Clu48]; [KU696405.1 Clu Can6, KX898329.1 Clu50]; [KU696410.1 Clu Can11]; [KU696409.1 Clu Can10]; [KU696408.1

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Figure S3. Minimum spanning tree haplotype network showing mitochondrial control region haplotypes (405 bp) using the randomized minimum spanning tree method (Paradis 2018).





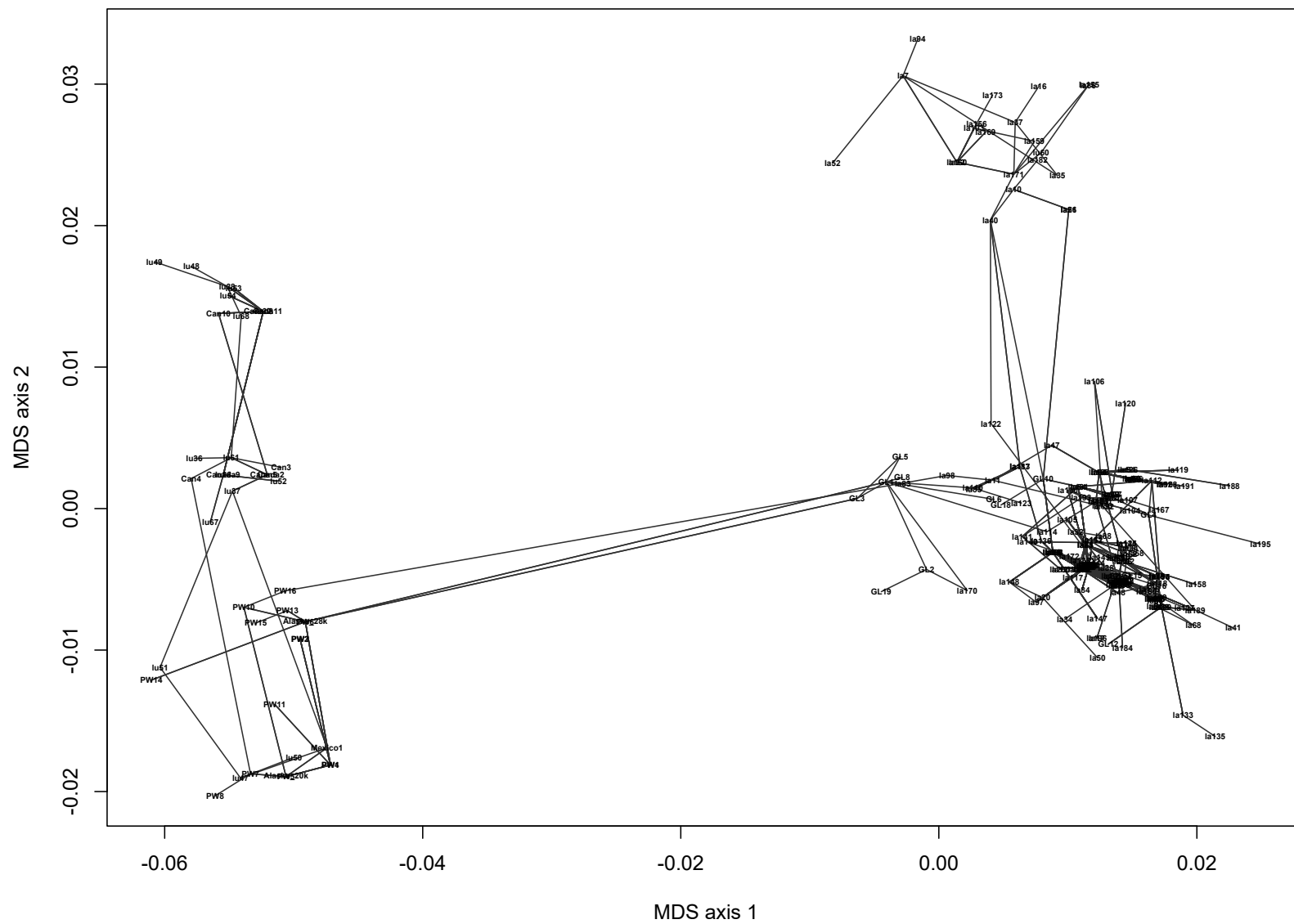


Figure S3