



Brief Communication

## Mitochondrial DNA Variation in Southeastern Pre-Columbian Canids

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### Abstract

The taxonomic status of the red wolf (*Canis rufus*) is heavily debated, but could be clarified by examining historic specimens from the southeastern United States. We analyzed mitochondrial DNA (mtDNA) from 3 ancient (350–1900 year olds) putative wolf samples excavated from middens and sinkholes within the historic red wolf range. We detected 3 unique mtDNA haplotypes, which grouped with the coyote mtDNA clade, suggesting that the canids inhabiting southeastern North America prior to human colonization from Europe were either coyotes, which would vastly expand historic coyote distributions, an ancient coyote–wolf hybrid, or a North American evolved red wolf lineage related to coyotes. Should the red wolf prove to be a distinct species, our results support the idea of either an ancient hybrid origin for red wolves or a shared common ancestor between coyotes and red wolves.

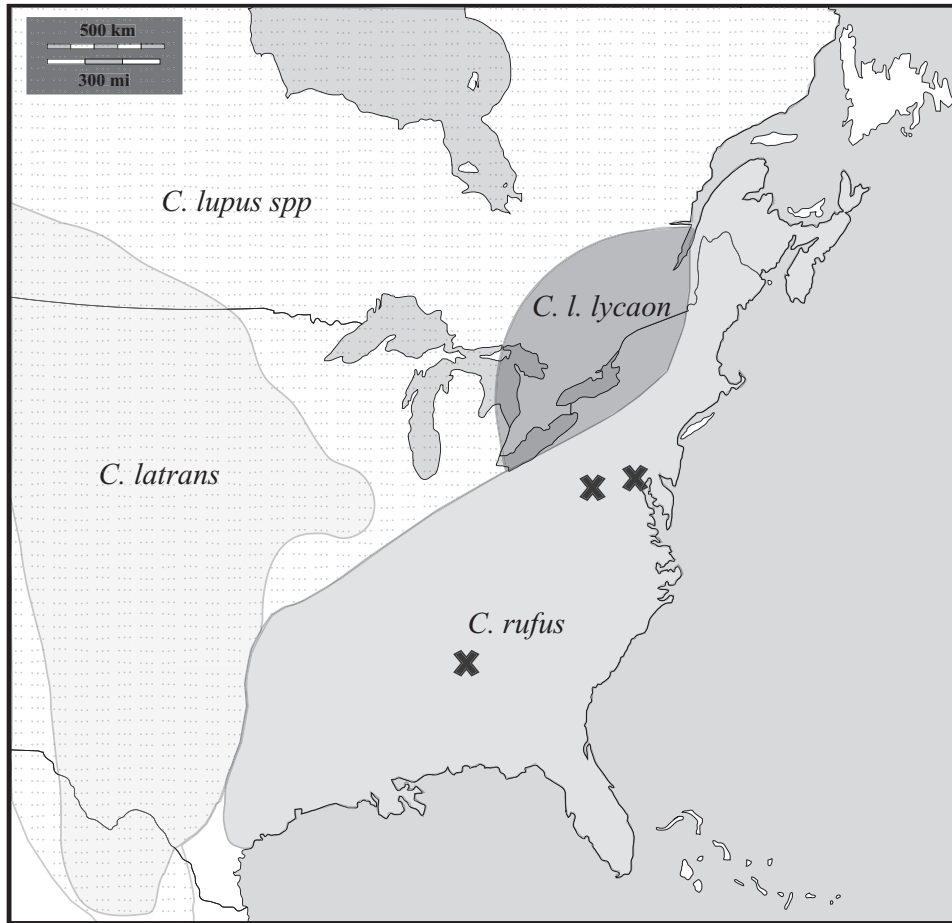
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**Key words:** ancient DNA, *Canis latrans*, *Canis rufus*, coyote, hybrid, red wolf.

The taxonomic status of eastern wolves in North America has been debated over many years (Nowak 1979, 1992, 2002; Wayne and Jenks 1991; Roy et al. 1994, 1996; Nowak and Federoff 1998; Wayne et al. 1998; Wilson et al. 2000; Murray and Waits 2007; vonHoldt et al. 2011; Chambers et al. 2012; Rutledge 2015), yet this debate has yielded little consensus on species delimitations. One hypothesis proposes that the eastern United States was historically inhabited by a wolf-like canid that experienced serious population declines following human colonization from Europe (Goldman 1937; Wilson et al. 2000; Nowak 2002; Chambers et al. 2012) via anthropogenic habitat degradation and extermination programs, which also facilitated the spread of coyotes eastward (*Canis latrans*; Parker 1995). Together with population declines, these factors may have caused extirpation and hybridization among various canid

populations in the east (Wayne and Jenks 1991; Lehman et al. 1991; Hailer and Leonard 2008; Rutledge et al. 2010; Figure 1).

At the center of the eastern canid species debate is the endangered red wolf (*Canis rufus*), a putative southeastern wolf species that currently persists in one small, reintroduced population in North Carolina (Hinton et al. 2013). Red wolves may have evolved as a distinct lineage in North America from a coyote-like ancestor (Nowak 1992, 2002; Nowak et al. 1998; Chambers et al. 2012) and may be conspecific with eastern wolves (*C. lycaon* or *C. lupus lycaon*), another controversial wolf species found primarily in Algonquin Provincial Park and adjacent areas in Ontario (Wilson et al. 2000; Kyle et al. 2006; Benson et al. 2012; Rutledge et al. 2012; Wilson et al. 2012; Rutledge et al. 2015). Alternatively, red wolves may represent a hybrid between gray wolves and coyotes (Wayne



**Figure 1.** Historic map of North American *Canis* species and approximate sampling locations (Xs) for ancient DNA samples. Distributions are based on [Parker \(1995\)](#), [Nowak \(2002\)](#), and [Chambers et al. \(2012\)](#); for alternative range distributions see [Kyle et al. \(2006\)](#) and [Rutledge et al. \(2010\)](#).

and [Jenks 1991](#); [Roy et al. 1994, 1996](#)), possibly appearing only within the last 430 years, i.e. since the European invasion of North America ([vonHoldt et al. 2011](#)).

Hybridization with coyotes, overlapping ranges with other canids, and small population size have contributed to the taxonomic confusion of red wolves ([Wayne and Jenks 1991](#); [Adams et al. 2003a](#); [Wilson et al. 2003](#); [Hailer and Leonard 2008](#)). Yet identifying distinct lineages is important for implementation of the Endangered Species Act, which does not have a clear rule for the management of recent hybrids ([Allendorf and Luikart 2007](#)). Understanding the evolutionary origins and historic distribution of eastern canids is also broadly important for wolf conservation in the United States. For instance, the conservation of Great Lakes wolves, a unique population of wolves in the Great Lakes region of the United States, Ontario, and Quebec, was jeopardized when gray wolves were delisted from the endangered species list in 2012, which removed protection for Great Lakes wolves until a 2014 federal court decision relisted them as a distinct population ([USFWS 2014](#)). The historic range and taxonomic status of Great Lakes wolves, which likely hybridized with eastern wolves and/or coyotes ([Koblmüller et al. 2009](#); [Wheeldon et al. 2010](#)), was a critical aspect of the initial controversial delisting of gray wolves ([Morell 2014](#), [NCEAS 2014](#)). Similarly, the Red Wolf Recovery Program recently underwent an intensive review in which the taxonomic status of the red wolf was once again questioned ([Wildlife Management Institute 2014](#)).

Examining the identity of canids found in the historic red wolf range prior to population declines and contemporary hybridization is critical to understanding how disturbance and biogeographic processes led to the contemporary canids now found in the southeastern United States ([Rutledge et al. 2010](#)). For instance, if red wolves are the result of coyote–gray wolf hybridization within the last 500 years, gray wolves and coyotes would have needed to have inhabited some portion of the southeastern United States during the pre-Columbian period. Current interpretations of the archeological record suggest that coyotes were absent from the southeastern United States between 10 000 and 100 years ago ([Parker 1995](#); [Nowak 2002](#)). However, there are records of coyotes as far east as West Virginia from over 10 000 years ago ([Parker 1995](#); [Nowak 2002](#)) and evidence of coyote range shifts in northeastern North America 11 000–8000 years ago ([Wilson et al. 2003](#); [Koblmüller et al. 2009](#); [Rutledge et al. 2010](#)). These range shifts were associated with habitat changes following the last glacial maximum, which also isolated gray wolf populations to the north and south of glaciers, and likely contributed to sub-speciation in western and northern North American gray wolves during the terminal Pleistocene ([Nowak 1995](#); [Leonard et al. 2007](#)). Canid range expansions during warm periods and subsequent range contractions during the glacial and interglacial periods may have been caused by changes in prey availability, habitat, and competition with larger canids ([Nowak 1995](#); [Vila et al. 1999](#)). For instance, the appearance of a medium-sized

wolf-like canid in the archeological record around 10 000 years ago in the southeast may have contributed to the exclusion of smaller coyotes (Berger and Gese 2007) from the southeastern United States (Nowak 2002).

Although the paleontological record suggests the presence of a wolf-like canid distinct from coyotes and gray wolves, and continuously present in the southeastern United States since the terminal Pleistocene (Nowak 2002), putative ancient wolf samples have not been genetically evaluated in the southeast. Given the paucity of genetic data used to clarify the taxonomic status of canids present prior to broad landscape changes, extirpation, and hybridization, we examined historic genetic samples from the southeastern United States. Specifically, we analyzed 3 canid DNA samples from the pre-Columbian period to assess the identity of the southeastern canid lineage.

## Methods

We analyzed 3 putative wolf teeth from within the historic red wolf distribution (Table 1; Figure 1), ranging in age from 350 to 1900 years old. The teeth were considered late-Woodland period and were aged by the type of faunal assemblages and early human activity present (CM 038379) (Guilday et al. 1962; Guilday 1982), carbon dating of associated human remains (CM 0006548) (Jackson 1987), or stratification and early human activity (UMI 91100) (Futato and Solis 1983) at their respective sites. Morphological analyses previously conducted on all 3 samples identified the teeth as red wolf teeth based on their significantly different size to homologues in both coyotes and gray wolves (Table 1 in Nowak 2002). Our criteria for selecting teeth for genetic analyses included assuring they were wolf-like in size, from the historic red wolf range, and were dated prior to contemporary canid population declines.

We conducted all DNA extractions and polymerase chain reaction (PCR) set-up in a genetics lab dedicated exclusively to ancient DNA (aDNA) analyses. DNA was isolated from teeth following the column-based aDNA extraction method outlined in Rohland et al. (2010). Prior to DNA extraction, we submerged all tooth samples in 6% bleach for 15 min to remove possible contaminants from the external surface (Kemp and Smith 2005), and manually ground samples to a fine powder with liquid nitrogen and a mortar and pestle cleaned with bleached and distilled water. We treated reagents and consumables following Champlot et al. (2010). We placed all tubes (clear-walled), PCR strips, water, rabbit serum albumin, and buffer within 1 cm of UV bulbs and irradiated them under UV light for 15 min. We treated dNTPS and Qiagen Hotstart *Taq* (Qiagen, Inc., Valencia, CA) with heat-labile double-strand specific DNase (Biotec Marine Biochemicals, Tromsø, Norway). We targeted the

mitochondrial DNA (mtDNA) control region, previously found to have a unique red wolf haplotype (Adams 2003a), with 4 primer pairs that generated overlapping sequences. The resulting amplicons were concatenated to produce a 450 base pair sequence (Leonard et al. 2002; Supplementary Table 1 online). We sent PCR product to Beckman Coulter Genomics (Danvers, MA) for bi-directional Sanger sequencing.

To ensure sequence reliability, we extracted DNA from every sample in 2 independent extractions and amplified and sequenced each DNA extract at least 4 times with all 4 primer pairs (Supplementary Table 2 online); we included several negative controls in every extraction and PCR to monitor contamination. We cloned and sequenced amplicons from 2 primer pairs for each putative wolf sample to detect DNA damage or potential contamination (Pääbo et al. 2004), and sent PCR product to MClab (San Francisco, CA) for cloning and sequence verification. Sequences were edited and compared with Sequencher v5.0; replicate DNA extractions were treated as independent samples and then compared for consistency before creating a final concatenated sequence for each individual. If there were ambiguous sites, we considered them resolved when 2 additional PCR reactions, overlapping sequences from flanking primer pairs, or cloning confirmed a base. All 3 mtDNA sequences were deposited in Genbank (Accession numbers: in progress).

We aligned our aDNA sequences with mtDNA control region sequences previously published on GenBank using the MUSCLE algorithm (Edgar 2004) implemented in Geneious v8.1 (Kearse et al. 2012). Comparison sequences included species our samples could represent: domestic dogs (*Canis lupus familiaris*), eastern wolves, gray wolves, Great Lakes wolves, red wolves, and coyotes (Supplementary Table 3 online). We used red fox (*Vulpes vulpes*) as the outgroup because the length of an available control region sequence (accession number AM181037) maximized the number of nucleotides in the full alignment, as opposed to poorly overlapping sequences from more closely related Ethiopian wolf (*Canis simensis*) or Golden jackal (*Canis aureus*). Similarly, some eastern wolf sequences were substantially shorter than the full alignment; to accommodate sequence length differences we ran 2 gene tree analyses, one with full sequence alignments of 370 basepairs, and a second with eastern wolf sequences but sequence alignments of 234 basepairs. We estimated the mtDNA control region gene tree using Bayesian and maximum likelihood (ML) methods from alignments including and excluding the outgroup sequence. In BEAST v1.8.2 (Drummond et al. 2012), we estimated a gene tree using the constant size coalescent tree prior and an uncorrelated lognormal relaxed molecular clock. We used a random starting tree, allowing the root of the tree to be one of the parameters that BEAST estimates. Two independent Markov Chain Monte Carlo (MCMC) analyses were

**Table 1.** Accession number, museum, age (years before present; ybp), material sampled, state and county location, and local site for ancient DNA specimens sampled for mitochondrial DNA (GenBank Accession KU556390-KU556392)

Accession number	Museum	Age (ybp)	Material sampled	Collection location	Archaeological site
CM 038379	Carnegie Museum of Natural History	350	Premolar	Pennsylvania, Lancaster County	Eschelman site
UMI 91100	University of Michigan Museum of Zoology	1000	Canine	Alabama, Jackson County	Crow Island Indian midden
CM 0006548	Carnegie Museum of Natural History	1900	Premolar	Pennsylvania, Bedford County	New Paris Sinkhole No. 2

run for 25 million steps, sampling every 2500 steps. We determined convergence on the posterior distribution by viewing the log files in Tracer v1.6. Convergence on the posterior is attained when the effective sample size (ESS) of a parameter (i.e. the number of effectively independent draws from the posterior distribution) is at least 200. All parameters in our analyses had ESS values greater than 300. We combined tree files in LogCombiner v1.8.2 with a 10% burnin for each file and calculated the maximum clade credibility (MCC) tree for the combined tree file in TreeAnnotator v1.8.2. We estimated a ML tree, performed 1000 bootstrap replicates, and calculated the 50% majority rule consensus tree using the GARLI v2.1 web service (Bazin et al. 2014).

## Results

All 3 ancient canid samples had unique mtDNA haplotypes (450 bp) not previously described. The validity of the aDNA sequences was supported by: 1) their similarity to modern canid haplotypes (no new indels or transversions); 2) no detected contamination in extraction negatives, PCR negatives, or cloning, and; 3) PCR and cloning replicates that were either identical or sufficiently consistent across most sequences to resolve questionable sites (Supplementary Table 2 online). There were, however, 5 ambiguous sites in CM 038379, 3 ambiguous sites in UMI 91100, and 3 ambiguous sites in CM 0006548 that we were unable to resolve through replicate PCR or cloning. Unresolved sites were all pyrimidine ambiguities (cytosine or thymine) suggesting there was some DNA damage caused by deamination of cytosine, a common issue with aDNA (Hofreiter et al. 2001). Our 3 aDNA sequences were unique no matter which base was used at ambiguous sites. We kept the degenerate base code in all analyses.

There were 2 well-supported clades in both the Bayesian and ML gene trees, irrespective of sequence length (Figure 2). One clade contained the domestic dog and gray wolf sequences plus 1 coyote sequence (AF541876) hypothesized to be from a coyote-dog hybrid (Adams et al. 2003b). The second clade contained all of the eastern, Great Lakes, and red wolf sequences, the remaining coyote sequences, and 1 gray wolf sequence (AY812740) believed to be a Mexican gray wolf-coyote hybrid (Leonard et al. 2005). These 2 mtDNA clades, Old World gray wolf/dog and New World coyote/red wolf/eastern wolf/Great Lakes wolf, are well supported in the literature (Roy et al. 1996; Vila et al. 1999; Leonard et al. 2002; Adams et al. 2003a; Hailer and Leonard 2008; Fain et al. 2010; Rutledge et al. 2010). Also similar to other studies, several eastern wolf haplotypes formed their own cluster within the New World clade (Figure 2B; Rutledge et al. 2010). The 3 novel aDNA sequences generated in this study grouped in the New World clade. Nodal support within these 2 clades was generally low, as is expected for closely related taxa, but 2 of the aDNA samples were sister to each other with moderate support (0.92 posterior probability, 0.76 bootstrap support). Of the 3 ancient teeth we evaluated, these 2 samples were the furthest apart geographically (Lancaster County, PA, and Jackson County, AL).

## Discussion

We detected 3 novel aDNA haplotypes that clearly grouped with the New World canid clade, rejecting the hypothesis that gray wolves were dominant in the southeast 500–2000 years ago. Within the New World mtDNA clade, the sequences we generated did not group closely with extant red wolf mtDNA haplotypes or the unique Algonquin eastern wolf cluster, suggesting they are not part of a

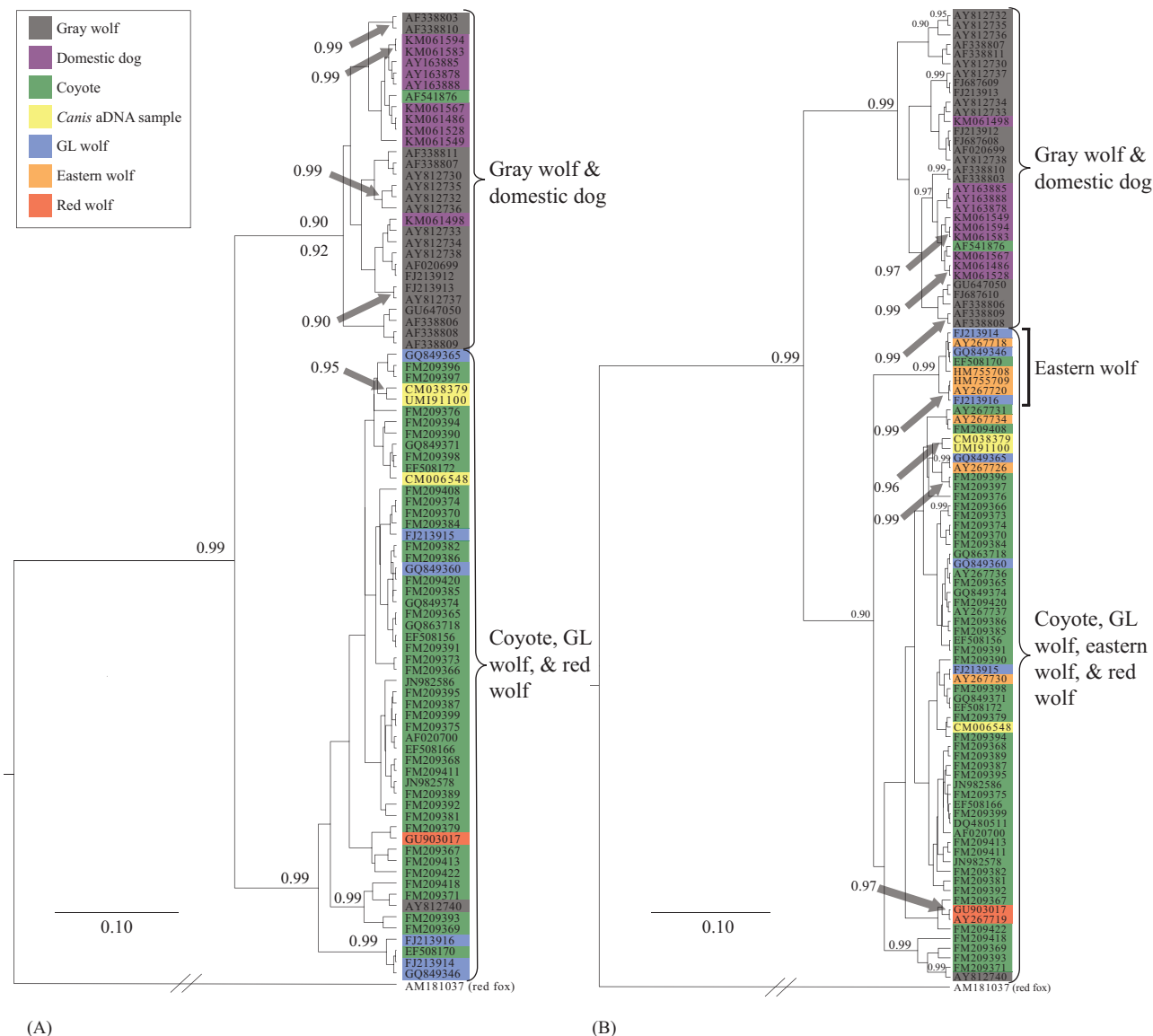
monophyletic eastern canid lineage. The 2 aDNA samples sister to each other grouped closely with haplotypes found in multiple other eastern canids, including northeastern Great Lakes wolves, eastern wolves, and southeastern coyotes (Hailer and Leonard 2008; Leonard and Wayne 2008; Rutledge et al. 2010), although nodal support was low. Similarly, the third novel aDNA haplotype clustered within coyote haplotypes found throughout the southeastern United States. This lack of geographic structure is consistent with other canid mtDNA studies that document little phylogenetic structuring of coyotes or gray wolves, a probable outcome of their high mobility (Vila et al. 1999; Koblmüller et al. 2012).

There are 3 plausible origins for the haplotypes we identified. First, our sequences could be from coyotes, which would indicate that coyotes were present in the southeastern United States continuously instead of intermittently as previously suggested. Although coyotes could have been present in the southeastern United States 350–1900 years ago, the size of the 3 teeth samples we analyzed was more wolf- than coyote-like (Nowak 2002). If morphological analyses are correct, it is more likely the teeth samples we analyzed represent a wolf.

Given the size of the teeth we sampled, the haplotypes we recovered may alternatively be the result of historic hybridization between coyotes and a wolf species (gray or red), leading to introgression of coyote haplotypes into the southeastern United States wolf population (Roy 1996). A similar scenario was observed both in eastern wolves (Wilson et al. 2003; Wheeldon and White 2009; Rutledge et al. 2010) and Great Lakes wolves (Koblmüller et al. 2009), which hybridized with coyotes when coyotes expanded their range during Pleistocene glacial and post glacial periods, resulting in coyote mtDNA haplotypes in modern wolf populations. If the ancient samples we analyzed here represent coyote-gray wolf hybridization events, some degree of hybridization occurred in the southeastern United States earlier than 287–430 years ago, as proposed by vonHoldt et al. (2011). Additionally, given the age of our samples (350–1900 years old), historic hybridization would likely have been due to natural events or early human activities, not landscape changes associated with European colonization. Under these circumstances, coyote-wolf hybrids may have occupied the southeastern United States for a long time, filling an important niche as a large predator (Roy et al. 1996).

If our samples represent an ancient coyote-red wolf hybridization event, it would also suggest coyote-red wolf hybridization has been a continuous and likely dynamic process up to the present day. Interestingly, canid hybridization is often unidirectional (i.e., female coyotes mate with male wolves; Lehman et al. 1991), which may explain why coyote mtDNA is found in putative eastern and red wolf populations and generally not the other way around. Yet with contemporary red wolves, females and males both hybridize with coyotes, although it may still be biased toward female coyotes (Hailer and Leonard 2008; Bohling and Waits 2015; Hinton et al. 2015), complicating hybridization patterns between the 2 groups. Additional analyses focused on Y-chromosome or nuclear genes in ancient samples would provide information regarding the paternal lineage (Hailer and Leonard 2008; Wilson et al. 2012; Bohling and Waits 2015), but such comparisons were beyond the scope of this study given the limited quantity and degraded quality of the aDNA.

Lastly, our historic samples could represent red wolves, a lineage that may be closely related to coyotes (Wilson et al. 2000; Hedrick et al. 2002; Chambers et al. 2012). Incomplete lineage sorting may explain why mtDNA haplotypes from ancient red wolves cluster with coyote mtDNA haplotypes, and not closely with extant red



**Figure 2.** Gene tree showing the relationships among canid mitochondrial control region sequences. Bayesian posterior probabilities above 0.90 are listed above the branches. Each color represents a different species: gray wolf (*C. lupus*), dog (*C. lupus familiaris*), coyote (*C. latrans*), Great Lakes wolf (*C. lupus/C. lupus lycaon*), eastern wolf (*C. lycaon/C. lupus lycaon*), and red wolf (*C. rufus*). Tip names indicate the Genbank accession number assigned to each sequence. The ancient DNA sequences generated in this study are named according to their museum accession numbers as in Table 1. (A) Gene tree based on sequence lengths of 370 basepairs, and (B) is a gene tree based on sequence lengths of 234 basepairs which include shorter eastern wolf sequences.

wolves. If coyotes and red wolves diverged from a common ancestor (Chambers et al. 2012), our aDNA sequences may represent shared ancestral haplotypes that have since been lost from contemporary red wolves, a possible result of population bottlenecks and inbreeding (Brzeski et al. 2014). Incomplete lineage sorting is common in recently diverged populations and species, and prevents reciprocal monophyly (Degnan and Rosenberg 2009). Others have observed this pattern within the Old World gray wolf/domestic dog clade (Vila et al. 1999; Leonard et al. 2002), as we have observed in the present study. There are other examples of distinct mammal species displaying paraphyletic mtDNA phylogenies, such as brown bears (*Ursus arctos*), polar bears (*Ursus maritimus*; Cronin et al. 1991), mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*; Cronin et al. 1988). While our study is limited in geographic scope and sample size, it is possible that our data indicates relatively

recent divergence between red wolf and coyote rather than hybridization. However, distinguishing incomplete lineage sorting from other hypotheses such as hybridization is difficult and requires more data than we have collected here.

Based on our results, red wolves may represent an evolutionary unit of conservation value, either as an ancient hybrid or as a unique lineage (Allendorf et al. 2001). These data suggest that a contemporary hybrid event was not the origin of red wolves. Hybridization is recognized as a natural evolutionary process and a facilitator of speciation (Mallet 2007); if red wolves have an ancient hybrid origin, it would not preclude the species from protection, and furthermore, it emphasizes the dynamic nature of canid evolution. If red wolves are a unique, independent lineage, they represent the only endemic wolf species in the United States, a species that is currently on the brink of extinction in the wild. Additional historic samples from a larger

geographic area will certainly help to clarify canid taxonomy in the southeastern United States, particularly if obtained sequences align closely with red wolves or the ancient haplotypes presented here. In the meantime, any plans to remove protection for red wolves would be premature.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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