Tracing Eastern Wolf Origins From Whole-Genome Data in Context of Extensive Hybridization

Sibelle T. Vilaça, †,1,2 Michael E. Donaldson,†,1 Andrea Benazzo,†,2 Tyler J. Wheeldon,3
Maria Teresa Vizzari,2 Giorgio Bertorelle,2 Brent R. Patterson,3 and Christopher J. Kyle*,1,4

1Environmental and Life Sciences Graduate Program, Trent University, Ontario, Canada

2Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

3Ontario Ministry of Natural Resources and Forestry, Wildlife Research and Monitoring Section, Trent University, Ontario, Canada

4Forensic Science Department, Trent University, Ontario, Canada

†These authors contributed equally to this work.

***Corresponding author:** E-mail: christopherkyle@trentu.ca.

Associate editor: Dr. Rasmus Nielsen

Abstract

Southeastern Canada is inhabited by an amalgam of hybridizing wolf-like canids, raising fundamental questions regarding their taxonomy, origins, and timing of hybridization events. Eastern wolves (*Canis lycaon*), specifically, have been the subject of significant controversy, being viewed as either a distinct taxonomic entity of conservation concern or a recent hybrid of coyotes (*C. latrans*) and grey wolves (*C. lupus*). Mitochondrial DNA analyses show some evidence of eastern wolves being North American evolved canids. In contrast, nuclear genome studies indicate eastern wolves are best described as a hybrid entity, but with unclear timing of hybridization events. To test hypotheses related to these competing findings we sequenced whole genomes of 25 individuals, representative of extant Canadian wolf-like canid types of known origin and levels of contemporary hybridization. Here we present data describing eastern wolves as a distinct taxonomic entity that evolved separately from grey wolves for the past ∼67,000 years with an admixture event with coyotes ∼37,000 years ago. We show that Great Lakes wolves originated as a product of admixture between grey wolves and eastern wolves after the last glaciation (∼8,000 years ago) while eastern coyotes originated as a product of admixture between "western" coyotes and eastern wolves during the last century. Eastern wolf nuclear genomes appear shaped by historical and contemporary gene flow with grey wolves and coyotes, yet evolutionary uniqueness remains among eastern wolves currently inhabiting a restricted range in southeastern Canada.

Key words: **whole genomes, admixture, eastern wolf, red wolf, great lakes wolf, eastern coyote.**

Introduction

Genomic investigations continue to reveal how extensive hybridization events are among species, including a spectrum of mammals, and the role these events play in local adaptation (Moran et al. 2021) and speciation (e.g., cetaceans Árnason et al. 2018; Westbury et al. 2020, and primates Tung and Barreiro 2017). Increasingly, whole-genome studies combined with refined statistical analysis show ancient hybridization events were common, with introgressed loci derived from such hybridization events still segregating in the genomes of many present-day species (Huerta-Sánchez et al. 2014; Gopalakrishnan et al. 2018; Kuhlwilm et al. 2019). Distinctions between ancient and recent hybridization events are important as hybridization linked to anthropogenic influences is often viewed as a threat to parental species (Allendorf et al. 2001; Ottenburghs 2021; Vilaça et al. 2023). Such perspectives require balance, however, in situations where parental species fitness may be compromised by rapidly changing habitats and selective pressures. In such cases, hybridization may allow for rapid adaptation and aid genetic rescue, thus maintaining both biodiversity and ecosystem integrity despite concerns of genetically swamping the parental species (Lewontin and Birch 1966; Pfennig 2021; Heuertz et al. 2023).

North America is inhabited by phenotypically diverse groups of wolf-like canids that generally overlap in range and are known to hybridize extensively in some regions but not in others (vonHoldt and Aardema 2020). In southeastern Canada, there is an array of hybridizing wolf-like canids that include eastern wolves (*Canis lycaon*, sensu Wilson et al. 2000), Great Lakes wolves (*C. lupus x C. lycaon*, sensu Wheeldon et al. 2010), and eastern coyotes (*C. latrans* var., sensu Hilton 1978). In the mid-1970s, Kolenosky and Standfield (1975) described the range of morphological and ecological variation among "grey" wolves in Ontario, Canada to include: 1) an "Ontario" type occurring primarily in the northern and central boreal forests north of Lake Superior, since termed the Great Lakes wolf (Leonard and Wayne 2008); 2) an "Algonquin" type occurring primarily in the mixed coniferous-deciduous forests east of Lake Superior and Georgian Bay, since termed the eastern wolf (Wilson et al. 2000); and 3) a "Tweed" type occurring apparently in more human-impacted landscapes north of Lake Ontario, since termed the eastern coyote (Hilton 1978). Studies using molecular markers show that wolf-like canids in and around Algonquin Provincial Park ("Algonquin" type or eastern wolf) appear genetically distinct relative to both coyotes and grey wolves, whereas wolf-like canids outside the Algonquin Provincial Park region are more heavily impacted by hybridization with either coyotes ("Tweed" type or eastern coyote) or grey wolves ("Ontario" type or Great Lakes wolf) (Wheeldon 2009; Wilson et al. 2009; Rutledge et al. 2010; Benson et al. 2012; Heppenheimer et al. 2018). While previous studies have noted the genetic distinctiveness of eastern wolves, attempts to address the taxonomy and origins of these wolf-like canids have been more problematic, relying on a baseline of either: 1) a two-species model that includes coyotes (*C. latrans*) and grey wolves (*C. lupus*) and various hybrids of these two species (vonHoldt et al. 2011, 2016); or 2) a three-species model that includes eastern wolves, that may also comprise red wolves, as a distinct species, separate from coyotes and grey wolves (Wilson et al. 2000; Sacks et al. 2021). In the three-species model, eastern wolves are thought to act as a gene flow bridge between coyotes and grey wolves (Kyle et al. 2006; Rutledge et al. 2010, 2015). Resolving between these competing models has been controversial and perhaps too restricted from a temporal perspective as recent studies suggest both ancient and recent hybridization among these canids should be considered in distinguishing between these models (Sinding et al. 2018; Sacks et al. 2021; Wilson and Rutledge 2021).

Whole-genome sequencing studies that have included wolf-like canid samples from southeastern Canada (vonHoldt et al. 2016; Sinding et al. 2018) have concluded that eastern wolves are likely of hybrid origin. Specifically, genomic studies showed that eastern wolves and Great Lakes wolves, while forming a distinct genetic group, were intermediate to grey wolves and coyotes (Sinding et al. 2018). These data were taken to refute the hypothesis of eastern wolves as a separate species, and to suggest they were more likely the result of admixture between grey wolves and coyotes, yet with unclear timelines as to when hybridization gave rise to eastern wolves and Great Lakes wolves (Sinding et al. 2018). Historical hybridization of eastern wolves with grey wolves in the relatively distant past (Wheeldon and White 2009) and with coyotes in the relatively recent past (Rutledge et al. 2012; Wheeldon et al. 2013), compounded by contemporary hybridization of eastern wolves with both grey wolves and coyotes (Rutledge et al. 2010; Benson et al. 2012; Heppenheimer et al. 2018), has made it difficult to discern clear taxonomic distinctions and account for the diversity of present-day wolf-like canids. This lack of clarity makes effective conservation and management planning difficult, where depending on the taxonomy and origins of these wolf-like canids, and the timing and relative extent of hybridization among them, different levels of protection would be afforded to these entities (COSEWIC 2015; COSSARO 2016).

To contextualize current genetic assessments aiming to determine the origins of, and hybridization patterns among, present-day wolf-like canids, geological records show the continuous presence of grey wolves in North America for the past 300,000 years (Loog et al. 2020). Contemporary North American grey wolves may, however, be descendants of a Beringian grey wolf expansion that occurred ~25,000 years ago (ya), replacing all midcontinent North American grey wolves and causing an expansion of coyotes (Loog et al. 2020). Additional hypotheses include extant wolves diverging from Beringian wolves earlier as proposed by Pacheco et al. (2022). There is also genomic evidence to suggest that all extant wolf-like canids in North America have at least 10–20% coyote ancestry (Bergström et al. 2022), reflective of wolf/coyote hybridization events 80–100 Kya. While some interpretations have the Beringian grey wolf replacing a type of North American grey wolf (Loog et al. 2020), Sacks et al. (2021) based on mitochondrial DNA (mtDNA) from historical samples postulated the endemic North American wolf may have been the red wolf. These mtDNA data further suggest red wolves diverged from coyotes ~60 Kya, with their "purest" descendants now confined to the greater Algonquin Provincial Park region of central Ontario, Canada, where they are commonly referred to as eastern wolves (Sacks et al. 2021). The descriptions of the various wolf morphotypes by Kolenosky and Standfield (1975) and genetic findings regarding eastern wolf hybridization/"purity" in central Ontario (Wheeldon 2009; Wilson et al. 2009; Rutledge et al. 2010; Benson et al. 2012; Heppenheimer et al. 2018; Sacks et al. 2021) suggest this region is of particular interest in investigating interrelationships of North American wolf-like canids.

The main objective of our study was to target knowledge gaps in our understanding of the genetics and evolutionary history of eastern wolves. Given past works highlighting distinct genetic clustering of eastern wolves relative to grey wolves, coyotes, and other wolf-like canids, we aimed to further identify what components of the eastern wolf genome were distinct from grey wolves and coyotes and better assess the timing of hybridization events among the wolf-like canid groups in northeastern North America. We used several analytical approaches to determine the proportion of ancestry unique to eastern wolves and we used novel statistical methods to assess the timing of hybridization events. Our work was conducted in context of both the two-species model that identifies the grey wolf and coyote as distinct species giving rise to various hybrids, including the eastern wolf, red wolf, Great Lakes wolf, and eastern coyote; and the three-species model that identifies the grey wolf, coyote, and eastern wolf (including red wolf) as distinct species giving rise to specific hybrids, including the Great Lakes wolf and eastern coyote. To address critiques of previous genomic studies (per vonHoldt et al. 2016; Hohenlohe et al. 2017), we sequenced new samples of known origin and levels of contemporary hybridization, with higher whole-genome coverage, and used more representative samples of Canadian canid types as appropriate outgroups (including pure groups of "western" coyotes and grey wolves sampled in Alberta).

Results

Population Structure

We sequenced 25 canid genomes (average coverage: 18.2×; range of coverage: 14.8× to 21.1×; supplementary table S1, Supplementary Material online) from Canada, including five individuals of each of the following canid types: Alberta "western" coyotes, Ontario eastern coyotes, Ontario eastern wolves from Algonquin Provincial Park, Ontario Great Lakes wolves, and Alberta grey wolves. We combined these new canid genomes with previously published canid genomes (vonHoldt et al. 2016; Gopalakrishnan et al. 2017; Sinding et al. 2018) for a total of 68 genomes in the analyzed dataset (fig. 1, supplementary table S1, Supplementary Material online).

Phylogenetic network analyses using all nuclear SNPs show eastern wolves form a separate group independent from grey wolves and coyotes (fig. 2*a*). Similarly, red wolves form a separate group, but are genetically closer to coyotes than grey wolves (fig. 2*a*). Great Lakes wolves and eastern coyotes are genetically closer to grey wolves and coyotes, respectively (fig. 2*a*). Fst statistics (fig. 2*b*, supplementary table S2, Supplementary Material online) show low differentiation within coyotes and within grey wolves, except for Mexican wolves, that were highly differentiated from other grey wolves. Eastern wolves had the lowest pairwise Fst values with Ontario Great Lakes wolves (Fst = 0.11), followed by Ontario eastern coyotes (Fst = 0.15) and Alberta grey wolves (Fst = 0.16). Admixture analyses were run using two datasets: the entire dataset with low and high coverage genomes ($n = 67$), and a reduced dataset with only higher coverage genomes (>10×, $n = 46$). When including low coverage genomes, the $K = 3$ subdivision supports separate clusters for grey wolves, coyotes, and eastern wolves, the $K = 4$ subdivision supports separate clusters for North American grey wolves and Eurasian grey wolves, and the $K = 5$ subdivision supports a separate cluster for red wolves (supplementary fig. S1, Supplementary Material online). It should be noted that Great Lakes wolves and eastern coyotes both clustered separately at higher values of K. Admixture analysis with the entire dataset reflects population subdivisions observed in previous studies, although no finer signals of population structure or admixture were detected in eastern wolves and red wolves (supplementary fig. S1, Supplementary Material online). When using only high coverage genomes (>10×), eastern wolves show admixture with grey wolves and coyotes, in greater and lesser proportions, respectively (fig. 2*c*), with optimal K by Evanno being $K = 4$ (supplementary fig. S2, Supplementary Material online). Further, eastern wolves have a unique ancestry component not shared with grey wolf or coyote populations outside of the Great Lakes region (fig. 2*c*). Red wolves also have a unique ancestry component and show admixture with coyotes and grey wolves, in greater and lesser proportions, respectively (fig. 2*c*). Great Lakes wolves have a mixed ancestry derived primarily from grey wolves, with considerable admixture from eastern wolves and limited admixture from coyotes (fig. 2*c*). Eastern coyotes have a mixed ancestry derived primarily from coyotes, with considerable admixture from eastern wolves and limited admixture from grey wolves (fig. 2*c*).

Population Dynamics and Genetic Diversity

Demographic history analysis using the multiple sequentially Markovian coalescent (MSMC2) showed effective population size (*Ne*) fluctuations throughout the evolutionary history of North American canids, with the lowest levels of *Ne* in all populations reached within the last few thousand years (fig. 3*a*). Before the start of the last glaciation (∼120,000 ya), a decline in *Ne* was observed across canid groups, followed by stabilization at the beginning of the glaciation and a separation of trajectories indicating partially independent demographic histories. While *Ne* continued to decline for some canid groups (e.g., Alberta coyotes, Alberta grey wolves, Arctic wolves, Yellowstone wolves), we observed a slight increase in *Ne* followed by a sharp decline for other canid groups (e.g., eastern wolves, red wolves, Great Lakes wolves, and eastern coyotes). In fact, eastern wolves had a higher *Ne* during the last glaciation than coyote and grey wolf populations.

Estimates of individual-levels of inbreeding (Fis) and heterozygosity show Canadian wolf-like canids, compared to other North American canids, have higher levels of heterozygosity and lower levels of inbreeding (fig. 3*b*). Most notably, eastern wolves and Great Lakes wolves have the lowest levels of inbreeding among all individuals, possibly a consequence of contemporary gene flow between them and with eastern coyotes and grey wolves, respectively. In Canada, "western" coyotes have on average higher levels of inbreeding than other canids, although grey wolves have on average lower levels of heterozygosity than other canids. These results demonstrate that "western" coyotes have higher genetic diversity than grey wolves, but also higher inbreeding coefficients. We did not find any recent shared identity-by-descent ancestry among the samples used in this study (supplementary fig. S3, Supplementary Material online, supplementary table S3, Supplementary Material online). Considering the total homozygous regions along the genome (F_{ROH}) (supplementary table S1, Supplementary Material online), red wolves and grey wolves had the highest F_{ROH} values,

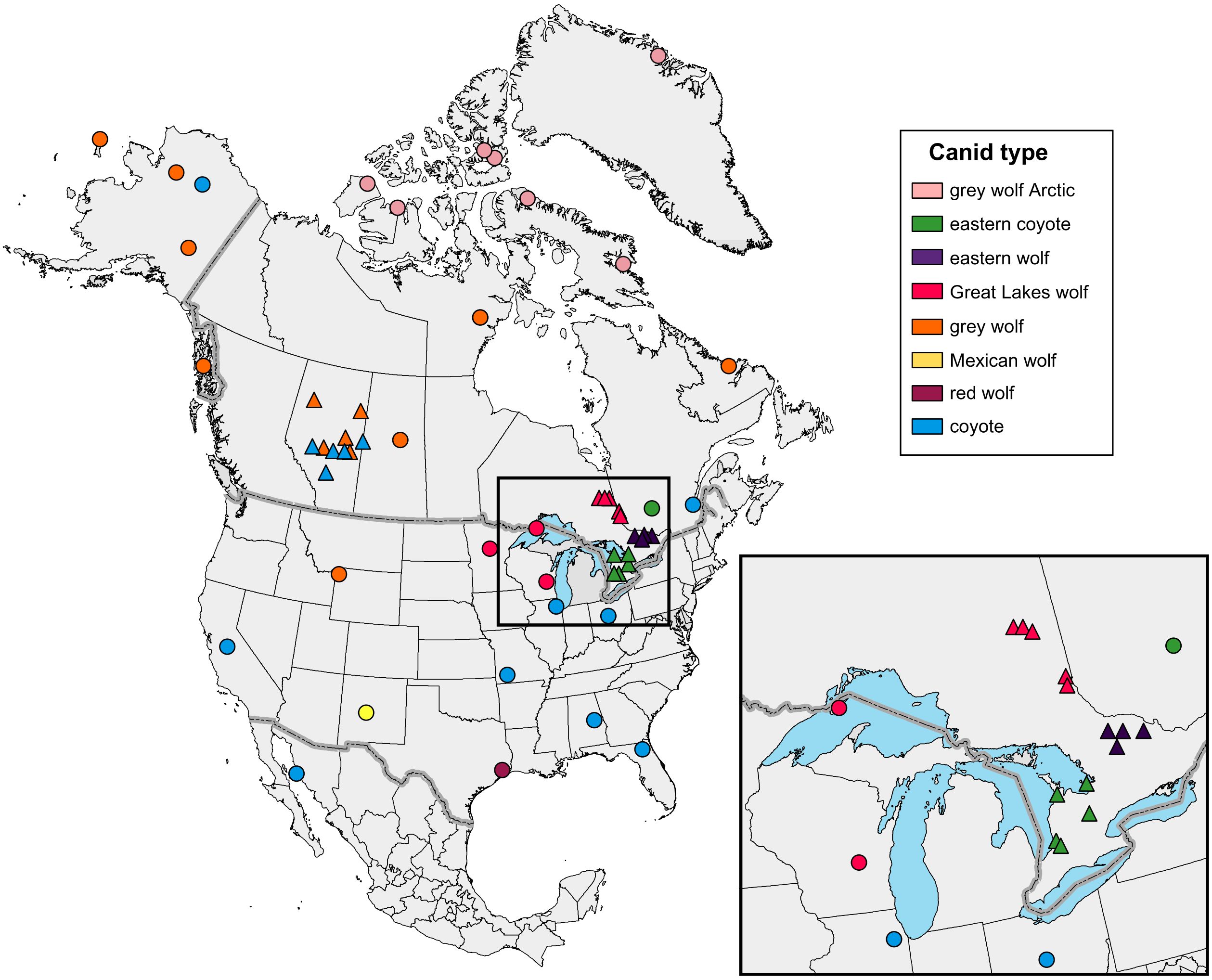

Fig. 1. Sample design for canid whole-genome sequence analysis. Triangles refer to samples newly sequenced in this study, while circles denote samples from the literature. The four Eurasian grey wolf samples and one golden jackal sample used as outgroups are not shown. The red wolf samples are placed according to vonHoldt et al. (2016) corresponding to captive breeding facilities in North Carolina, however, the original capture zone for the founders is approximately Texas/Louisiana (Sinding et al. 2018). Inset map highlights wolf-like canid samples from the Great Lakes region. Map layers were obtained from Commission for Environmental Cooperation Atlas.

whereas "western" coyotes and eastern coyotes had the lowest F_{ROH} values. Even though the F_{ROH} estimates were different between the two methods used here, they were highly concordant (Pearson correlation coefficient = 0.91).

Past and Recent Gene Flow Between Canid Groups

To investigate past gene flow between canid groups we used a time-dependent estimate of migration rates between pairwise populations (MSMC-IM). The highest migration rates were between eastern wolves × eastern coyotes and eastern wolves × Great Lakes wolves (fig. 4). This indicates gene flow between eastern wolves and other canid groups in the Great Lakes region with higher migration rates in the last 2,000 years (fig. 4*a*). Considering the cumulative migration probabilities ($M_{(t)}$, fig. 4*b*), a plateau is also observed between eastern wolves × eastern coyotes and eastern wolves × Great Lakes wolves. This pattern indicates the amount of ancestry that has merged through past migration events and can be interpreted as a conservative estimate of past admixture proportions. While the plateau can also be observed in other combinations with grey wolves, these are older (~5,000 ya) than the plateaus with Great Lakes wolves and eastern coyotes. Therefore, past gene flow with grey wolves involved more archaic grey wolf groups and with larger contributions to the genome [indicated by $M_{(t)}$ proportions]. We also notice that for Great Lakes wolf and eastern coyote combinations, $M_{(t)}$ never reached 0 (i.e., complete separation), a pattern explained by continuous gene flow between these groups. However, eastern coyotes are a recent group (see below and fig. 5) and estimates of migration rates with eastern wolves precede their presumed origin in Ontario. Therefore, we interpret past gene flow observed between eastern wolves and eastern coyotes, specifically before the latter's origin during the last century, as contemporary eastern coyotes serving as a proxy for the "western"

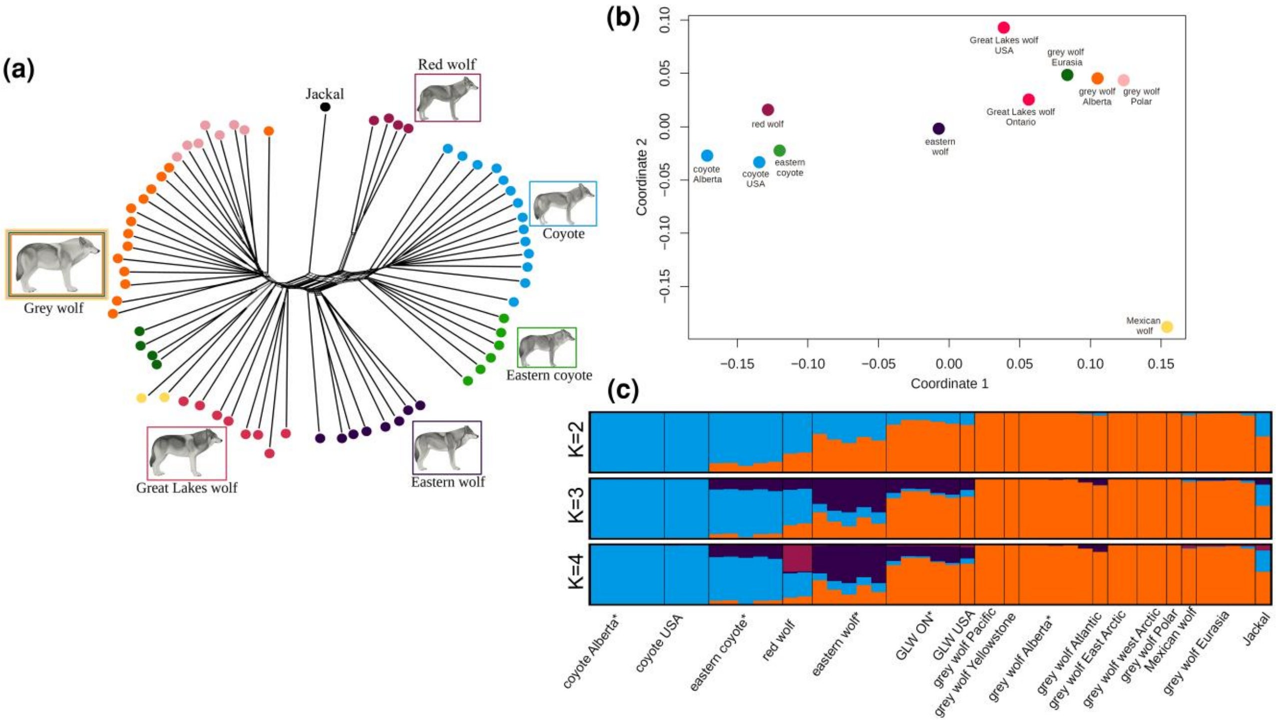

Fig. 2. (*a*) Splitstree network for 6,020,173 autosomal SNPs. Distances were expressed as "1-IBS". (*b*) Multidimensional scaling (MDS) analysis of pairwise Fst values. (*c*) Admixture plot showing the estimated ancestry proportions of 46 individuals determined using Structure for $K = 2$ through $K = 4$ based on only high coverage genomes (>10×; 42,785 autosomal SNPs from 50 kbp windows). Each partitioned vertical bar represents an individual's proportional membership to the inferred populations. Asterisks next to sample names indicate samples sequenced in this study. $K = 5–9$ not shown, yielding no further biologically relevant partitions. GLW = Great Lakes wolf.

coyotes that hybridized with eastern wolves. Similarly, past gene flow observed between eastern wolves and Great Lakes wolves, specifically before the origin of Great Lakes wolves after the last glaciation, is interpreted in the context of contemporary Great Lakes wolves serving as a proxy for the grey wolves that hybridized with eastern wolves.

To explicitly test the two- versus three-species models and elucidate the evolutionary history of the eastern wolf, we assessed these models using approximate Bayesian computation via random forest (ABC-RF). We designed our models to infer if eastern wolves had a hybrid origin (grey wolf × coyote hybrid) or derived from a single group with putative posterior admixture (i.e., incorporation of a new genetic background into an existing lineage; supplementary fig. S4, Supplementary Material online). If they had a hybrid origin, the estimated time of divergence between a separation from grey wolves and coyotes would show an overlapping posterior distribution (assuming hybrid mixing is not asymmetrical). Otherwise, the origin from one group would have an earlier date than the subsequent admixture. Furthermore, our models allowed the admixture proportion to be close to 0 (supplementary table S4, Supplementary Material online). Posterior estimates for this admixture parameter close to 0 indicate a single origin without posterior admixture.

The model with higher posterior probability (Model 2, posterior probability = 0.76; supplementary table S5, Supplementary Material online) inferred the eastern wolf derived from a grey wolf population (fig. 5). The estimated posterior distributions for all parameters can be found in the supplementary materials (supplementary tables S4 and S6, Supplementary Material online), along with the confusion matrix (supplementary table S7, Supplementary Material online) and the corresponding densities (supplementary fig. S5, Supplementary Material online). The divergence time between coyotes and grey wolves was estimated as 914,619 ya (95% Confidence Interval [CI]: 820,493–1,047,184; $r^2 = 0.11$) (fig. 5), compatible with previous estimates (Wilson et al. 2000; Wilson and Rutledge 2021). The separation of eastern wolves from grey wolves occurred 66,595 ya (95% CI: 19,746–281,139; $r^2 = 0.61$) (fig. 5). Our results show the ancestral eastern wolf population has been evolving separately from grey wolves for ∼67,000 years, and during this time developed population-specific ancestry as observed in Structure and NGSadmix results (fig. 2*c*, supplementary fig. S1*b*, Supplementary Material online). Following this divergence, an admixture event with coyotes occurred 37,601 ya (95% CI: 7,721–164,817; $r^2 = 0.89$) (fig. 5). Great Lakes wolves originated as a product of admixture between grey wolves and eastern wolves after the last glaciation (7,899 ya; 95% CI: 1,176–18,918; $r^2 = 0.24$) (fig. 5). Eastern coyotes originated as a product of admixture between "western" coyotes and eastern wolves, but the timing of this admixture event could not be confidently estimated ($r^2 = 0.01$), likely reflecting recent (i.e., ∼100

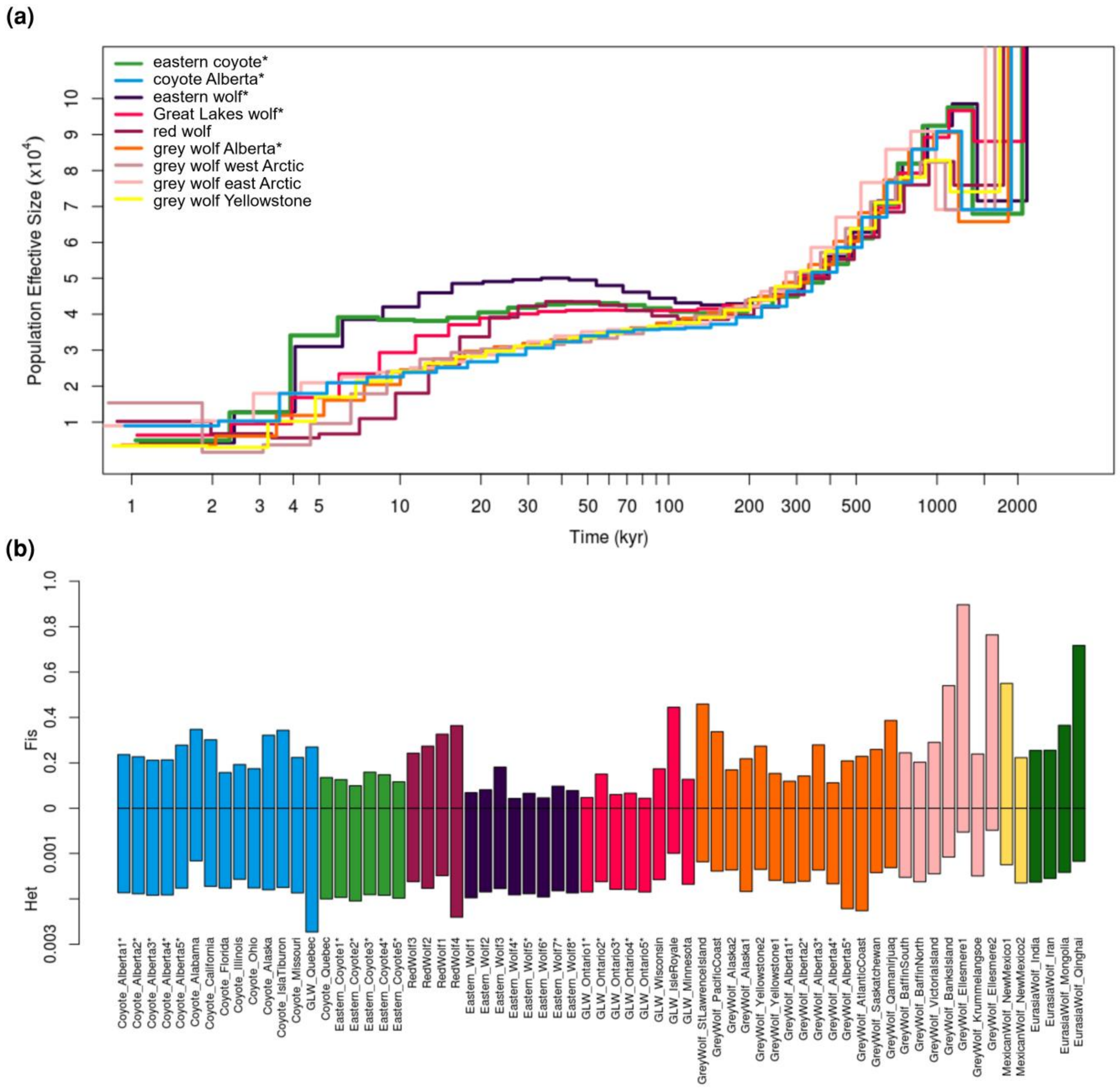

Fig. 3. (*a*) MSMC2 population size estimates from four haplotypes per population. (*b*) Bar charts of heterozygosity estimates (Het) and inbreeding coefficients (Fis) per individual. Asterisks next to sample names indicate samples sequenced in this study. GLW = Great Lakes wolf; kyr = thousands of years in the past.

ya) coyote colonization of eastern North America and concomitant hybridization with eastern wolves and domestic dogs (Kyle et al. 2006; Wheeldon et al. 2013; Hody and Kays 2018). Given the complex demographic scenario, not all demographic parameters could be accurately estimated, including migration rates that showed low r^2 values ($r^2 \leq 0.10$, supplementary table S4, Supplementary Material online). Finally, we used a posterior-predictive check to assess if the selected model was able to generate the observed genetic diversity by comparing the simulated and observed data through a principal component analysis (supplementary fig. S6, Supplementary Material online). The most supported model of our ABC-RF analysis, and the estimated values of its parameters, was able to reproduce the genetic variation pattern found in the observed data (supplementary fig. S5, Supplementary Material online).

The best-fitting graph estimated using AdmixtureBayes (supplementary fig. S7, Supplementary Material online) supported the selected model from the ABC-RF analysis with concordant admixture events and proportions. The topology with the highest posterior probability (PP = 0.38, fig. 5*b*) showed that eastern wolves originated from an early admixture event, after the first split between coyote and grey wolf lineages (fig. 5*b*, supplementary fig. S8, Supplementary Material online). This ancestral admixed group has a larger proportion of wolf than coyote ancestry (58% wolf × 42% coyote), similar to the ABC-RF estimates (54% wolf × 46% coyote,

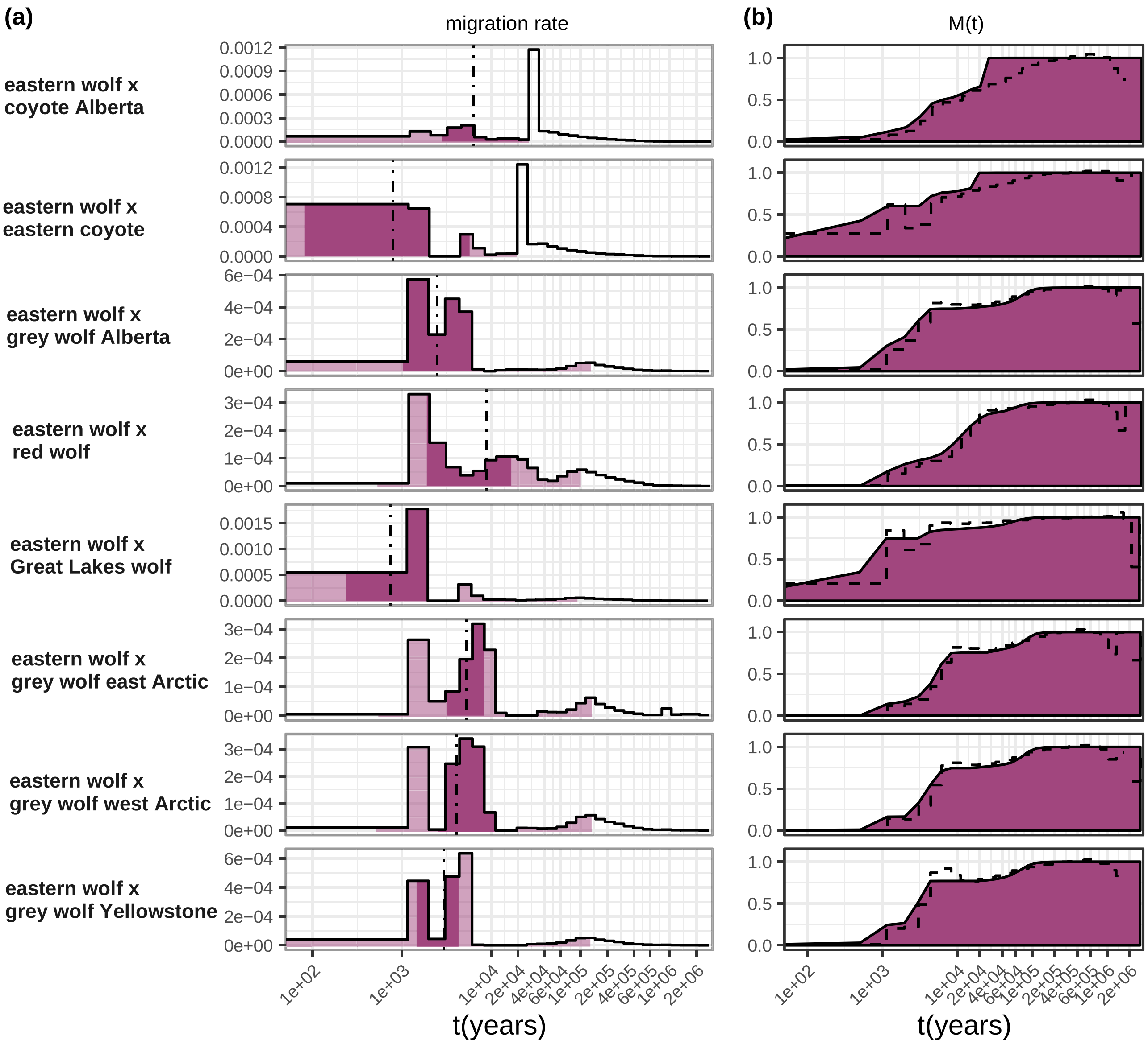

Fig. 4. Migration profiles from MSMC-IM between eastern wolves and other canid groups. (*a*) Timing and dynamics of separation process between two groups visualized by the time-dependent-symmetric migration rate $m(t)$. Time is represented as years in the past. The dashed lines represent the median, or the time when 50% of the ancestry between the two groups merged. Shading indicates 1–99% (lighter shade) and 25–75% (darker shade) percentiles of the cumulative migration probabilities. (*b*) Cumulative migration probabilities that estimate the proportion of ancestry already merged at time t, and represents proportions of gene flow through time. $M_{(t)}$ values close to 0 denote complete separation between the two groups, while 1 shows a complete mix as one population. Dashed lines represent the relative cross-coalescent rate.

supplementary table S4, Supplementary Material online). Great Lakes wolves and eastern coyotes originated from more recent admixture events, with ancestry proportions compatible with the ABC-RF and Structure analyses (supplementary table S4, Supplementary Material online, fig. 2*c*). While both Great Lakes wolves and eastern coyotes have eastern wolf components, they derive from two different ancestral groups along the eastern wolf evolutionary branch (nodes n2 and n4 in fig. 5*b*, supplementary figs. S8 and S9, Supplementary Material online). This result supports the older origin of Great Lakes wolves containing eastern wolf ancestry that may be distinct from the current genetic composition of eastern wolves.

Patterns of Gene Flow Across the Genome

To investigate differential ancestry patterns, admixture history, and differential evolution across the genomes of eastern wolves, we used the "chromosome painting" method implemented in *Chromopainter* (Lawson et al. 2012) to identify local ancestry along the genome and summarize information about ancestral relationships. Almost two-thirds of the eastern wolf genome is specific to this group. On average, an eastern wolf individual had its genome "painted" as 63% eastern wolf, 19% grey wolf, and 18% coyote (supplementary table S8, Supplementary Material online). We estimated the pairwise genetic distance of the fragments identified as eastern wolf ancestry with

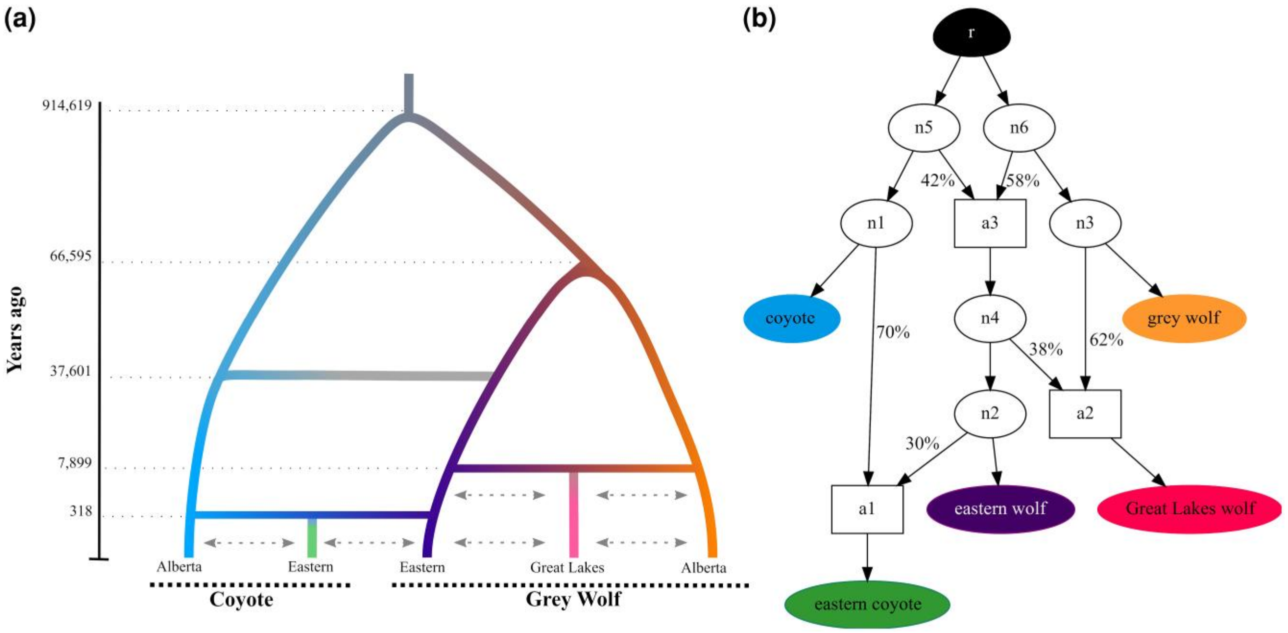

Fig. 5. (*a*) Most supported model of ABC-RF analysis. Numbers on the left represent point (median) estimates of divergence times. (*b*) Admixture graph topology with highest posterior probability using AdmixtureBayes. Tip nodes indicate the sampled genomes used to fit the graph that are the same as those for the ABC-RF analysis. Percentage numbers on the branches represent admixture proportions. Convergence graphs for AdmixtureBayes analysis can be found in supplementary figures S10 and S11, Supplementary Material online.

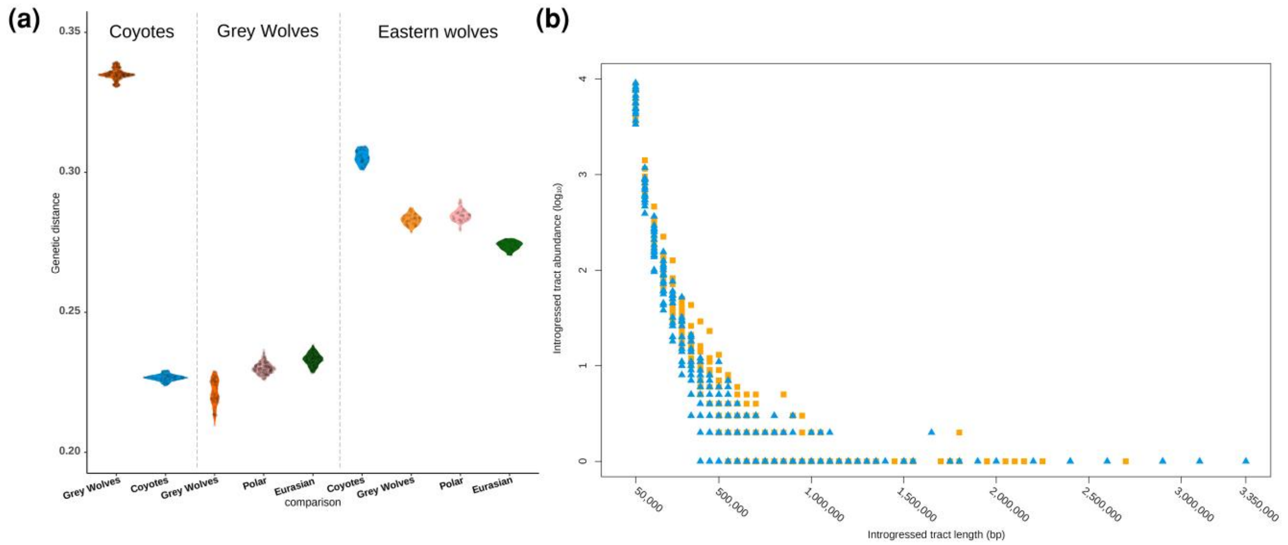

Fig. 6. (*a*) Genetic distance of the fragments painted as eastern wolf ancestry with other canid populations. (*b*) Observed distributions of introgressed fragments for eastern wolf individuals. Orange and blue represent introgressed fragments from grey wolves and coyotes, respectively. The abundance of introgressed fragments as a function of their length is represented for all eight eastern wolf individuals. Polar = grey wolf Polar; Eurasian = grey wolf Eurasia.

other canid populations (fig. 6*a*). Our results show eastern wolves have a lower genetic distance to Eurasian grey wolves than to other North American canid populations (fig. 6*a*).

We also extracted the length of chromosome fragments assigned to one of the three reference groups (i.e., eastern wolf, grey wolf, coyote), as this measure might inform if introgressed fragments are derived from ancient or recent gene flow (short and long, respectively). Within eastern wolves (fig. 6*b*, supplementary fig. S12, Supplementary Material online), the average length of fragments painted as eastern wolf ancestry was longer than that of introgressed fragments painted as grey wolf or coyote ancestry (Eastern $\text{Wolf}_{\text{LENGTH}} = 80.90$ kbp; Grey $\text{Wolf}_{\text{LENGTH}} = 40.11$ kbp; $\text{Coyote}_{\text{LENGTH}} = 34.94$ kbp). However, considering only introgressed fragments (fig. 6*b*), the maximum length of those painted as coyote ancestry was longer than that of those painted as grey wolf ancestry ($\mathrm{Coyote}_{\mathrm{MAX}} = 3.31$Mb; Grey $\mathrm{Wolf}_{\mathrm{MAX}} = 2.69$Mb), showing that in some eastern wolves, the introgression of coyote fragments is more recent than that of grey wolf fragments.

Discussion

Herein, we assess the evolutionary history of eastern wolves and the timing of hybridization events among wolf-like canid groups in northeastern North America in the context of the two- versus three-species models of North American wolf-like canid taxonomy. Our attempt to address these competing models differs from previous studies in that we contextualized our analyses with 1) grey wolf and coyote samples from populations known to have no contemporary hybridization to serve as outgroups, and 2) eastern coyote and Great Lakes wolf samples with known levels of contemporary hybridization. Further, we used several analytical approaches to determine the proportion of ancestry unique to eastern wolves (beyond that there were unique characters per Heppenheimer et al. 2018), and we used novel approaches (i.e., ABC-RF, AdmixtureBayes) to assess the timing of hybridization events. Several lines of evidence support the hypothesis that eastern wolves are a distinct taxonomic entity, with population-specific ancestry, derived from a grey wolf population present in North America before the Last Glacial Maximum. Despite access to few representatives published genomes, our analyses show red wolves also display a unique ancestry component but are more impacted by contemporary gene flow with coyotes than eastern wolves. We also found contemporary eastern wolves show evidence of ancient and recent gene flow with coyotes and grey wolves similar to other recent studies (Sacks et al. 2021; Wilson and Rutledge 2021; Bergström et al. 2022). Our results, with the inclusion of low coverage genome data, yielded similar equivocal conclusions to previous studies using the same data and analyses to address the competing two- versus three-species models (vonHoldt et al. 2016; Sinding et al. 2018). However, when using only high coverage genomes, we show eastern wolves and red wolves both have private genomic signatures and evidence of admixture with grey wolves and coyotes. The use of genomes with higher coverage is important when no pure representatives are available (e.g., eastern wolves with no contemporary admixture), as analysis such as NGSadmix with low coverage genome data might mask patterns of admixture in structured populations like eastern wolves (Skotte et al. 2013). While lower coverage genomes have played an important role in other studies (Lou et al. 2021), when estimating gene flow without reference to nonadmixed populations higher coverage genomes should be given preference. Another key finding from our study includes the estimation of ancestral population dynamics showing larger effective population sizes (*Ne*) for both ancestral coyote and grey wolf populations followed by a bottleneck after the LGM when Beringian grey wolves colonized North America. These results suggest North American canids were deeply impacted by the last glaciation, probably due to a combination of low temperatures and less habitat that caused population bottlenecks and replacements (Loog et al. 2020). The colonization of the Americas by modern humans, and competition for large game, are also postulated to have contributed to *Ne* decreases in these canid populations (Fan et al. 2016). We cannot rule out, however, that the ancestral *Ne* values were higher based on new lineages entering the genome from admixture events thought to have occurred during the last ice age (e.g., Sacks et al. 2021; Wilson and Rutledge 2021).

What is an Eastern Wolf?

Previous studies have used fossils, mtDNA, microsatellite markers (see Chambers et al. 2012), reduced representation genomic data (Heppenheimer et al. 2018), and most recently whole-genome data to evaluate competing hypotheses regarding the taxonomic status of eastern wolves considered as either a hybrid of coyotes and grey wolves (e.g., vonHoldt et al. 2016; Sinding et al. 2018) or a distinct species (Wilson et al. 2000). Using a multifaceted combination of analyses, we show that to an extent, both hypotheses are compatible with our results. From genome-wide analyses, eastern wolves appear to be intermediate between coyotes and grey wolves (figs. 2*a* and 2*b*). In more refined analysis, however, eastern wolves show a unique ancestry component, with evidence of both ancient and recent admixture with coyotes and grey wolves (figs. 3*b*, 5, and 6). Thus, eastern wolves have mosaic genomes with unique ancestry components despite ancient and recent admixture with coyotes and grey wolves. This pattern is exemplified by Chromopainter analyses that show private eastern wolf genomic regions are intercalated with short and long introgressed fragments from coyotes and grey wolves. Short introgressed fragments are a signal of ancient gene flow, while long introgressed fragments are a signal of recent gene flow (Duranton et al. 2018). Consequently, our results support an independent evolutionary lineage of eastern wolves post-divergence from grey wolves, followed by ancient and recent admixture with coyotes and grey wolves. The genomes of contemporary eastern wolves inhabiting the Algonquin Provincial Park region appear to be a mosaic between an ancient eastern wolf population that survived in eastern North America and both grey wolves and coyotes.

Based on whole-genome data, the most supported model from ABC-RF and admixture graph analyses (fig. 5) indicate eastern wolves are a distinct taxonomic entity, yet derived from grey wolves. Eastern wolves appear to have differentiated from grey wolves by approximately 67 Kya, admixed with coyotes by approximately 38 Kya, and maintain contemporary gene flow with both coyotes and grey wolves. We interpret the simulated model as concordant with observations of unique eastern wolf fragments being more similar to grey wolves than coyotes (fig. 6*a*). Given eastern wolves have evolved separately from grey wolves for the past ~67,000 years, they have had sufficient time to accumulate polymorphisms distinguishable from other canids.

Previous studies based on mtDNA and Y-chromosome data suggested eastern wolves are a distinct species closely related to coyotes, and thus evolved from a New World canid lineage (Wilson et al. 2000, 2012; Sacks et al. 2021; Wilson and Rutledge 2021). Characteristic mtDNA and Y-chromosome haplotypes of eastern wolf origin cluster within the coyote clade, but separately therein, and comprise divergent haplotypes that are not observed in non-hybridizing coyote populations (Wilson et al. 2000, 2012). Such data are seemingly at odds with autosomal data that suggest eastern wolves are a distinct taxonomic entity derived from grey wolves, and thus evolved from an Old World canid lineage. The admixture event between eastern wolves and coyotes ~38 Kya (or more ancient events per Bergström et al. 2022) plausibly explains the presence of coyote-like mtDNA and Y-chromosome haplotypes in eastern wolves that comprise a divergent lineage (Wilson et al. 2000, 2012). After this admixture event, the ancient eastern wolf population possibly “inherited” coyote clade mtDNA and Y-chromosome haplotypes, that subsequently diverged, and are now considered to be of eastern wolf origin (Wilson et al. 2000, 2012). Our estimates differ from the Wilson et al. (2000) divergence time of 150–300 Kya between eastern wolves and coyotes but align with mtDNA coalescence estimates based on sequences obtained from modern and ancient samples (Wilson and Rutledge 2021).

A genomic investigation of ancient canid samples by Bergström et al. (2022) found all North American wolf-like canids contain significant proportions of coyote-like ancestry, including populations with no contemporary hybridization with coyotes. Interestingly, they report two Pleistocene wolves from the Yukon had coyote-like mtDNA. Bergström et al. (2022) suggest that wolves and coyotes diverged ~700 Kya, with evidence of hybridization between coyotes and wolves from around 80–100 Kya; implying that Pleistocene coyotes had a more northern range than previously thought, or coyote/wolf admixture spread northward via wolf populations. That admixture occurred between grey wolves and coyotes 80–100 Kya, as postulated by Bergström et al. (2022), is within our 95% confidence interval for ABC-RF parameter estimates for eastern wolf divergence from grey wolf (fig. 5). Thus, our estimates for introgression of coyote haplotypes into the grey wolf population, from which the eastern wolf derived, are compatible with results based on ancient DNA (Bergström et al. 2022). This implies that the admixture event identified in our analyses ~38 Kya could have been the source of secondary introgressed haplotypes (i.e., Wheeldon and White 2009). This scenario, while speculative, implies that the unique eastern wolf genomic signature primarily reflective of grey wolf autosomal ancestry also included introgressed coyote autosomal ancestry (Rutledge et al. 2010; Wilson et al. 2012). Although we cannot reject that possible hybridization between eastern wolves and other ancient coyote forms that inhabited North America (e.g., *C. latrans orcutti*, Wilson and Rutledge 2021) could have been the source of coyote-like mtDNA and Y-chromosome haplotypes observed in eastern wolves (Wheeldon 2009, Rutledge et al. 2010; Wilson et al. 2012), the availability of more genome data from extinct canids (Perri et al. 2021) might soon enlighten these relationships.

Recent research has indicated that although grey wolf populations have been present in North America for the past 300,000 years, these populations may have been extirpated following the LGM. Therefore, these pre-LGM populations would not be represented in the genetic diversity of extant grey wolves (Loog et al. 2020) descendants from Beringian grey wolves that expanded through North America post-LGM and replaced indigenous Pleistocene grey wolf populations. During the LGM, North America was covered by ice sheets and canid populations likely retracted to southern regions, leaving northern areas free of canid presence (Koblmüller et al. 2016) or within Beringian refugia (Pacheco et al. 2022). In contrast, our results suggest that pre-Beringian grey wolf populations survived in eastern North America, and some pre-LGM grey wolf ancestry remains within the eastern wolf. This interpretation is corroborated by lower genetic distances between genome fragments of eastern wolves and Eurasian grey wolves. Therefore, eastern wolf genomic ancestry appears closer to that of Eurasian grey wolves than modern North American grey wolves.

While we suggest that eastern wolves appear to be a distinct taxonomic entity with population-specific ancestry, we do acknowledge that our interpretations of eastern wolf origins are mostly compatible with those from other recent genome studies. Bergström et al. (2022) and Sinding et al. (2018) found admixture between wolves and coyotes, with wolf ancestry derived post-LGM (vs. pre-LGM per our data) best explain their data but acknowledge other complex ancestries could explain observed patterns. Overall, describing the exact ancestral events that led to current day eastern wolves may be intractable without further analyses of ancient canid samples, yet we maintain that genomic signatures specific to eastern wolves best describe a distinct taxonomic entity with population-specific ancestry.

Red Wolves

Data from two high coverage red wolf genomes show a unique ancestry component different from that of eastern wolves, with evidence of substantial admixture with coyotes, but only limited admixture with grey wolves. Wilson et al. (2000) hypothesized that eastern wolves and red wolves are possibly the same species, and Sacks et al. (2021) suggested that eastern wolves are presently the “purest” representatives of the original red wolf. Our results demonstrate that both eastern wolves and red wolves have experienced recent admixture with other canids, but they still carry private ancestries in their genomes. Admittedly, interpretations from only two genomes limit the scope of interpretation from these data, where additional population-level high coverage genomes and historical samples (i.e., before the captive breeding program) are needed to better elucidate the evolutionary history of red wolves.

Great Lakes Wolves and Eastern Coyotes

Our analyses confirm the mixed ancestries of Great Lakes wolves and eastern coyotes, yet with differing taxonomic and temporal origins. Based on mtDNA and Y-chromosome data, Great Lakes wolves have been considered hybrids between grey wolves and eastern wolves (Wheeldon 2009; Fain et al. 2010; Wheeldon et al. 2010), and eastern coyotes have been considered hybrids between "western" coyotes and eastern wolves (Kays et al. 2010; Way et al. 2010; Wheeldon et al. 2013; Monzón et al. 2014). In contrast to past studies, whole-genome data (this study) suggest Great Lakes wolves and eastern coyotes are perhaps more appropriately considered distinct populations of grey wolves and coyotes, respectively, albeit affected by admixture with eastern wolves. We note limited coyote admixture in Great Lakes wolves and limited grey wolf admixture in eastern coyotes that likely results from bridging gene flow via the eastern wolf (Rutledge et al. 2010).

Great Lakes wolves likely originated ~8,000 ya, after the ice sheets retreated from the Great Lakes region around 9,000 ya (Dyke 2004). ABC estimates suggest Ontario Great Lakes wolves are more recent than previous estimates for Minnesota counterparts (7,899 vs. 27,000–32,000; vonHoldt et al. 2016). Previous studies have demonstrated Great Lakes wolf populations exhibit mtDNA and Y-chromosome haplotypes of both grey wolf origin and eastern wolf origin (Wheeldon 2009; Fain et al. 2010; Wheeldon et al. 2010). Our results suggest Great Lakes wolves have ancient origins from an admixture event between grey wolves and eastern wolves, but their ancestry is predominantly grey wolf. Accordingly, Great Lakes wolves are perhaps best described as a distinct population of grey wolves affected by ancient admixture with eastern wolves. Future studies should further investigate the origins and current dynamics of Great Lakes wolves that occupy an important ecological niche in the mixed and boreal forests of central Canada and the western Great Lakes states.

The eastern coyote originated too recently to be confidently dated via the simulated model. This finding is in agreement with observations that coyotes were rare in the Great Lakes region until ~1890 (but see Thiel 2020) and reached southern Ontario by 1919 (Hilton 1978; Parker 1995; Hody and Kays 2018). Natural history records indicate eastern coyotes originated during the last century as a consequence of hybridization events associated with the eastward range expansion of "western" coyotes facilitated by land clearing and removal of larger predators (Hilton 1978; Parker 1995; Hody and Kays 2018). Previous studies have found eastern coyote populations exhibit mtDNA and Y-chromosome haplotypes of both coyote origin and eastern wolf origin (Kays et al. 2010; Way et al. 2010; Wheeldon et al. 2013), and also Y-chromosome haplotypes of domestic dog origin (Wheeldon et al. 2013). While we acknowledge the lack of domestic dog samples included in our population structure and admixture analyses could bias our results with respect to eastern coyotes known to exhibit autosomal admixture from domestic dogs (Monzón et al. 2014), our results align with previous findings to suggest eastern coyotes have recent origins from admixture events between "western" coyotes and both eastern wolves and domestic dogs, yet maintain predominantly coyote ancestry. Accordingly, eastern coyotes could be described as a distinct population of coyotes affected by recent admixture with both eastern wolves and domestic dogs. Given the ancient and historical natures of grey wolf × eastern wolf hybridization and "western" coyote × eastern wolf hybridization, respectively, we suggest it is appropriate to refer to Great Lakes wolves and eastern coyotes as admixed as opposed to hybrids. We justify this interpretation by noting backcrossing to the parental canid type and subsequent isolation (at least within their core ranges) has occurred for both canid types.

Conclusion

In Canada, eastern wolves have been recognized as a distinct species (*C.* sp. cf. *lycaon*) based on genetic, morphological, and behavioral studies (COSEWIC 2015). Eastern wolves are listed as "Special Concern" in Canada under the federal Species at Risk Act (COSEWIC 2015) and "Threatened" in Ontario under the provincial Endangered Species Act (COSSARO 2016). Although eastern wolves have been recognized as a separate species from coyotes and grey wolves in Canada, they are currently managed as a single species together with eastern coyotes and Great Lakes wolves across their primary range. The pooling of these three taxonomic entities for management is considered necessary because of the difficulty in visually distinguishing between wild canids and their hybrids in central Ontario leading to challenges in enforcing hunting and trapping regulations. Using high coverage genomes and population-level analysis, we reconcile results from previous publications based on specific molecular markers. Eastern wolves are indeed a taxonomically distinct entity that has experienced both ancient and recent gene flow with grey wolves and coyotes. Considering our results, and per precautionary principles, eastern wolves may warrant continued conservation and protection as they maintain a portion of unique genetic ancestry. Ancient hybridization with both coyotes and grey wolves has influenced what comprises present-day eastern wolves, making it complicated to disentangle from recent hybridization. From what is presumed to have been a larger historical range, the "purest" forms of eastern wolves remain only in a small region of central Ontario and potentially southern Quebec. Regardless of the complex processes that led to the eastern wolf in its current form, there remains a unique eastern wolf genomic signature, and uncertainty regarding the evolutionary history of the eastern wolf should not be an impediment to its conservation.

Materials and Methods

Sample Collection

To create a better baseline of "pure" *C. lupus* and *C. latrans*, we obtained and sequenced five grey wolf samples and five "western" coyote samples from Alberta that represent "pure" reference samples for our analysis. We also obtained and sequenced five samples of each of the main wolf-like canid types in Ontario, including eastern wolves, Great Lakes wolves, and eastern coyotes. We selected samples for sequencing based on sex and background genetic data (i.e., autosomal microsatellite genotype) to identify as pure a representation of each wolf-like canid type as possible. Description of microsatellite genotyping can be found in Supplementary Text 1 and supplementary table S9, Supplementary Material online. Based on these analyses, we selected male samples of eastern wolves ($n = 5$), Great Lakes wolves ($n = 5$), and eastern coyotes ($n = 5$). Samples of eastern coyotes were taken from regions within their original range (i.e., southeastern Ontario) and samples of eastern wolves were taken from their core range within Algonquin Provincial Park, as those taken from the edge of their range outside Algonquin Provincial Park may be hybrids with Great Lakes wolves (to the north) or eastern coyotes (to the south). Details on laboratory procedures and genome sequencing can be found in supplementary text 1, Supplementary Material online.

Our total dataset comprised another 43 previously published canid genomes (vonHoldt et al. 2016; Gopalakrishnan et al. 2018; Sinding et al. 2018) comprising North American grey wolves ($n = 18$; including two Mexican wolves), Eurasian grey wolves ($n = 4$), coyotes ($n = 9$), eastern wolves ($n = 3$), red wolves ($n = 4$), Great Lakes wolves ($n = 4$), and one golden jackal (*Canis aureus*). We downloaded raw reads from the NCBI sequence read archive (the complete list of samples can be found in supplementary table S1, Supplementary Material online). Details on variant calling and filtration can be found in supplementary text 1, Supplementary Material online.

Population Structure and Admixture Analyses

We assessed population structure and admixture among different canid groups starting with the 6,020,173 autosomal SNPs from 68 individuals. Although our relatedness estimates did not show kinship among any of the used samples, to be conservative we excluded sample Yellowstone1 (SRR7976423) from population structure analysis due to conflicting reports of kinship with other Yellowstone samples (vonHoldt et al. 2016; Sinding et al. 2018). We estimated population clustering and ancestry proportions using NGSadmix (Skotte et al. 2013) testing values of K (number of population clusters) from $K = 1$ to $K = 20$, with 50 replicates. Only SNPs with minimum allele frequency greater than 0.05 were used for this analysis. A network tree was created with *SplitsTree* v4.13.1 (Huson 1998) using a distance matrix obtained with *PLINK* v1.90 (Purcell et al. 2007) and the 6,020,173 autosomal SNPs from 68 individuals. The distances were expressed as "1-IBS" and missingness correction was used. Population-based pairwise Fst was calculated using ANGSD and summarized through a Multidimensional scaling (MDS) using the *cmdscale* function in R v4.03 (R Core Team 2017). Additionally, we used *vcftools* v0.1.16 (Danecek et al. 2011) to select for individuals with a genome coverage $>10\times$. This yielded 46 individuals and 6,019,845 autosomal SNPs. Next, we thinned that dataset so that no two sites were within 50 kbp from one another, yielding 42,785 autosomal SNPs. We estimated population clustering and ancestry proportions using *Structure* v2.3.4 (Falush et al. 2003) with default settings (e.g., correlated allele frequencies model), running the admixture model 10 times at $K = 1$ through $K = 9$ for 2E + 5 iterations following an initial burn-in of 5E + 4 iterations. Results were evaluated using the online tool *CLUMPAK* vbeta (Kopelman et al. 2015). We assessed optimal K based on Delta K graphs (Evanno et al. 2005).

Heterozygosity, Inbreeding, Relatedness, and Runs of Homozygosity

SNP density was calculated as Watterson's Theta (Watterson 1975) with *ANGSD*. Inbreeding (Fis) and heterozygosity (Het) were estimated using ANGSD following Sinding et al. (2018). Relatedness across all samples was estimated using NgsRelate (Korneliussen and Moltke 2015) from genotype likelihoods to account for low coverage samples. We used parameter settings similar to Margaryan et al. (2020) with maximum likelihood estimates (−m 1), a log-likelihood difference lower than 10^{-6} between two consecutive EM-steps (*-t* 1e-06) as a stopping criterion for the EM algorithm, and a maximum number of steps of 10,000 (−*i* 10,000). Pairwise relatedness was assessed using the coefficients for noninbred relatives k_0, k_1, and k_2, which represent the proportions of the genome where two individuals share 0, 1, and 2 alleles identical by descent (IBD), respectively. To infer kinship among pairwise combinations of samples, we used the expected values: monozygous twins ($k_0 = 0$, $k_1 = 0$, $k_2 = 1$), parent-offspring ($k_0 = 0$, $k_1 = 1$, $k_2 = 0$), full-sibs ($k_0 = 0.25$, $k_1 = 0.5$, $k_2 = 0.5$); half-sibs, uncle-nephew or grandparent-grandchild ($k_0 = 0.5$, $k_1 = 0.5$, $k_2 = 0$).

Runs of homozygosity (ROHs) were calculated using BCFtools (Narasimhan et al. 2016), and with VCFtools considering 50 kbp windows. To calculate the proportion of genome in ROH (F_{ROH}), only ROH regions longer than 1 Mb were considered.

Population Dynamics and Migration

To estimate population dynamics through time and the timing of population separations, we used MSMC2 (Schiffels and Wang 2020, https://github.com/stschiff/msmc2). We used the phased callable regions of two individuals per population, totaling four chromosomes per population. Unphased SNPs and scaffolds shorter than

500 kbp were excluded as recommended (Schiffels and Wang 2020). Final graphs were scaled considering a generation time of 3 years and a substitution rate of 4×10^{-9} substitutions/site/year (vonHoldt et al. 2016). To obtain the migration rate and separation times between two population pairs, we used MSMC-IM (Wang et al. 2020), which uses cross-coalescent estimates from MSMC2 to calculate the time-dependent estimate of gene flow in two pairs of populations.

ABC Random Forest

To compare different evolutionary models and infer the evolutionary history of wolf-like canids in southeastern Canada, we used approximate Bayesian computation (ABC). ABC is a flexible framework to estimate the likelihood of complex models based on coalescent simulations. Until recently, due to computation power limitations, the use of ABC was restricted to a few markers. A recent extension of ABC, the ABC-random forest (ABC-RF), is based on machine learning and allows the computation of complex models using whole-genome data. ABC-RF requires fewer simulations per model, without sacrificing accuracy in estimating parameters or comparing models (Pudlo et al. 2016; Ghirotto et al. 2021). Based on our results, we simulated two alternative demographic models in which the only difference was the relationship of eastern wolves as an ancient divergent population derived from coyotes or grey wolves. Given previous knowledge based on natural history records (Hody and Kays 2018) and/or genetic data (vonHoldt et al. 2011), eastern coyotes were set as a product of recent admixture (<500 ya) between coyotes and eastern wolves, while Great Lakes wolves were set as a product of ancient admixture (<20,000 ya) between grey wolves and eastern wolves. We allowed gene flow between canid groups to account for known recent admixture (Kyle et al. 2006; vonHoldt et al. 2016; Sinding et al. 2018). For eastern wolves, we modeled an ancient divergence from either coyotes or grey wolves, followed by genetic drift, and an admixture event with either grey wolves or coyotes, respectively. The addition of a genetic drift step allowed us to account for the possibility that eastern wolves comprise a divergent population with a unique genetic signature (as shown by our Structure and NGSadmix analyses), but also to estimate if they are a product of hybridization between coyotes and grey wolves. In the case of hybridization, we expected the time between admixture (TxEW) and divergence from the source population (TdEW) to be an overlapping distribution between the two parameters (indicating they happened at approximately the same time). If they evolved as a separate population, the two distributions would be nonoverlapping, and we could estimate the time since eastern wolves were evolving as a separate population. Finally, the divergence between coyotes and grey wolves was based on that estimated from Wilson et al. (2000).

We summarized the genomic variation of our data by estimating the frequency distribution of four categories of segregating sites (FDSS; Wakeley and Hey 1997). To calculate the FDSS we considered the genome as subdivided into k independent fragments of length m, and for each fragment, we counted the number of segregating sites belonging to each of the four categories defined as 1) segregating sites private of the first population; 2) segregating sites private of the second population; 3) segregating sites that are polymorphic in both populations; and 4) segregating sites fixed for different alleles in the two populations. The resulting vector of summary statistics is composed of four frequency distributions (one for each category described above) for each pair of populations considered in the analysis. Each distribution describes the observed frequency of k genomic loci having exactly a certain number of segregating sites in the truncated space from 0 (monomorphic loci) to a fixed maximum of 40.

We ran 100,000 simulations per model, using ms (Hudson 2002). We generated 10 chromosomes per population (five individuals each), and 9,209 independent fragments of 500 bp length. To select the number of fragments, we estimated the highest number of contiguous fragments, separated by at least 10 kb, that were present in the observed data. The ms command line can be found in supplementary text 2, Supplementary Material online. The observed FDSS was calculated from the VCF file using scripts from https://github.com/anbena/ABC-FDSS including variants located in the 9,209 loci. Mutation rates, generation times (3 years), and recombination rates (1E-8) were based on (vonHoldt et al. 2016). All ABC-RF estimates were performed using the functions *abcrf* and *regAbcrf* from the R package *abcrf* (Pudlo et al. 2016; Raynal et al. 2019) and employing a forest of 5,000 trees. We compared all models and obtained the posterior probabilities using the function *predict*. To assess the quality of the model selection procedure we evaluated the confusion matrix and the classification errors (supplementary tables S5 and S6, Supplementary Material online). The quality of the parameter estimation procedure was assessed through the coefficient of determination (r^2). This coefficient represents the fraction of variance of the parameters explained by the summary statistics used to summarize the data, where an $r^2 < 0.10$ suggests the summary statistics do not have sufficient information about the parameter estimated (Neuenschwander et al. 2008).

Admixture Graph

To further support our ABC-RF models, we performed an admixture graph estimate using AdmixtureBayes (Nielsen et al. 2023). AdmixtureBayes is a Bayesian approach that uses a reversible jump Markov Chain Monte Carlo (MCMC) to find a best-fitting graph that describes the ancestry of the analyzed populations, not requiring a priori information (i.e., a tree topology) on populations splits or the number of admixture events. Graphs were estimated using the same higher-coverage samples as used in ABC-RF and a thinned dataset of SNPs sampled every 50 kbp to ensure independence between loci. The golden jackal was used as outgroup. We ran three independent

MCMC chains each consisting of 40,000,000 steps (*-n* 800000), discarded the first 50% as burn-in, and checked for convergence using the EstimateConvergence.R script (https://github.com/avaughn271/AdmixtureBayes). All other parameters were left as default.

Chromosome Painting

To explore admixture levels in our samples and infer portions of the genome unique to each of the canid groups (coyotes, grey wolves, and eastern wolves), we used *Chromopainter* (Lawson et al. 2012). To identify local ancestry, based on patterns of haplotype similarity of donor groups, eastern wolf individuals were "painted" using all high coverage samples as donors, and one eastern wolf was used as a recipient (leave-one-out procedure). The recipient eastern wolf chromosomes were reconstructed as a combination of DNA fragments from three donor groups (coyotes, grey wolves, and eastern wolves). The donor group of each genomic fragment was identified as the most similar haplotype from the reference populations. Therefore, the local ancestry profile of a given (recipient) eastern wolf chromosome was made of a mosaic of DNA fragments for which the probability to be inherited from either donor group was inferred by Chromopainter. In each independent run, one eastern wolf individual was a receiver and all high coverage genomes, excluding the recipient, were set as donors. Thus, eight independent runs were performed. We used a >80% threshold probability to assign a painted fragment to one of the donor groups. Regions having an assignment probability below the 80% threshold were considered as ambiguous. Regions were painted in 10 kbp intervals. To estimate if the fragments painted as eastern wolf ancestry were more closely related to grey wolves or coyotes, in each run, we extracted the regions of the genome that were painted as eastern wolf ancestry (i.e., private of eastern wolves). These same fragments were extracted in all individuals, and we calculated a pairwise genetic distance measurement between canid groups (coyotes, North American grey wolves, Arctic wolves, and Eurasian grey wolves). Genetic distance was calculated as allelic matches. For each SNP, genetic distance (D) equals 0 when two individuals are homozygous for the same allele (AA/AA), $D = 0.5$ when at least one of the genotypes in two individuals is heterozygous (Aa/AA, AA/Aa, or Aa/Aa), and $D = 1$ when two individuals are homozygous for different alleles (AA/aa or aa/AA). The distance is then standardized by the number of analyzed SNPs.

We also calculated the length of each painted fragment within each eastern wolf individual. To determine the start and end of each fragment, we considered a region as contiguous if it was flanked by a region of the same donor group and was interrupted by a single tract assigned as ambiguous or to a different donor group. We used *bedtools merge* to combine fragments. Intervals assigned to the same ancestry were then summed and plotted in R in bins of 50 kbp.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

This research was funded by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada, grant number RGPIN-2016-05373 (C.J.K.), and the Species at Risk Research Fund for Ontario, grant number RF_24_18_Trent7 (C.J.K. and B.R.P.). We thank Mark Ball (Government of Alberta) for providing samples of grey wolves and "western" coyotes from Alberta. We thank Matt Harnden (Natural Resources DNA Profiling and Forensics Centre at Trent University) for technical assistance and Sergio Pereira (The Centre for Applied Genomics at The Hospital for Sick Children) for performing the Illumina sequencing. We thank Ke Wang and Stephan Schiffels for helping with MSMC-IM, and Andrew Vaugh for suggestions on AdmixtureBayes. This research was enabled in part by support provided by Compute Canada (www.computecanada.ca; RRG gme-665-ab). Canid illustrations by Justine Lee Hirten and Jon Way for Eastern Coyote/Coywolf Research. We thank two anonymous reviewers and the associate editor for their helpful comments on an earlier draft of this manuscript.

Author Contributions

Conceptualization, C.J.K. and B.R.P.; analysis, S.T.V., M.E.D., A.B., M.T.V., and T.J.W.; resources, C.J.K. and B.R.P.; G.B. data interpretation; S.T.V. wrote the first draft and all authors contributed to the final version of the manuscript.

Data Availability

All high-throughput sequencing files are archived in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA641325.

Conflict of interest statement: The authors declare no competing interests.

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