



Perspective

Conservation genomics in perspective: A holistic approach to understanding *Canis* evolution in North America

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ABSTRACT

The use of genome-wide single nucleotide polymorphisms (SNPs) in wildlife conservation is growing rapidly and promises to revolutionize the field of population genetics. However, genomic data should not be interpreted in isolation of other biological and complementary genetic data. Here, we used recently published genome-wide SNP data from *Canis* species in combination with data from more traditional genetic markers and evidence from non-genetic fields to show how a broader frame of reference is important for interpretation of genomic data. We conducted a principal components analysis (PCA) and phylogenetic analysis of 48 K SNP data to show that a three-species model of *Canis* evolution in North America is not rejected by the data and deserves further investigation with more representative samples from Algonquin Park. We also demonstrate how a holistic perspective provides a more complete picture of evolutionary processes underlying diversification and speciation events in the genus *Canis*. Overall, we highlight the importance of careful examination and synthesis of all lines of evidence for the implementation of effective conservation policies for endangered species.

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1. Introduction

The use of genome-wide single nucleotide polymorphisms (SNPs) in assessing population structure in wildlife species has seen a marked increase in recent years. SNPs are an attractive marker for evolutionary studies because they occur throughout the

genome, they are bi-parentally inherited, they follow a relatively simple and well-defined pattern of mutation, genotypes are easily standardized across laboratories, and high-throughput technology allows thousands of loci to be analyzed for a relatively low cost (Morin et al., 2004). They should not, however, be viewed as an indiscriminate replacement for other molecular markers in the population genetics toolkit because SNPs may not be “unconditionally superior to more traditional approaches” in all circumstances (Schlötterer, 2004), particularly when sampling is inadequate and/or metadata is insufficient or misinterpreted such that erroneous conclusions are drawn.

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In a recent volume of *Genome Research*, vonHoldt et al. (2011) analyzed 48,036 SNPs in wolves, coyotes, and dogs to develop a better understanding of admixture in wild canid populations. The paper provides an extensive amount of genomic data that contributes significantly to the field of canine genetics. The data do not, however, support the authors' conclusion of a two-species model of *Canis* evolution in North America that relegates a third species, the eastern wolf (*Canis lycaon*) (referred to as "Great Lakes wolf" by the authors), to the product of hybridization between gray wolves (*C. lupus*) and coyotes (*C. latrans*). The incorrect conclusions drawn by the authors contribute to the perceived lack of consensus regarding wolf taxonomy that has led the United States Fish and Wildlife Service (USFWS) to retract its original recognition of the eastern wolf as a distinct species, *C. lycaon* (USFWS, 2011a,b) and continue to treat it as a gray wolf subspecies (*C. lupus lycaon*) (USFWS, 2012), despite the lack of scientific evidence to support

it as such. Here, we re-analyzed different subgroups of the 48 K SNP data and interpret the results within a broader genetic and ecological context. We conclude that a three-species model of *Canis* evolution that includes *C. lycaon* as a distinct North American evolved wolf is not rejected by the data and therefore deserves further investigation. Moreover, we demonstrate that the data presented by vonHoldt et al. (2011) are misconstrued due to flawed assumptions that bias the interpretation on various analytical levels, and because the authors fail to interpret their findings within the context of ecology, natural history, the fossil record, and other genetic markers.

2. Methods

We provide a critique of the methods, results, and conclusions of vonHoldt et al. (2011) and highlight points that need to be more

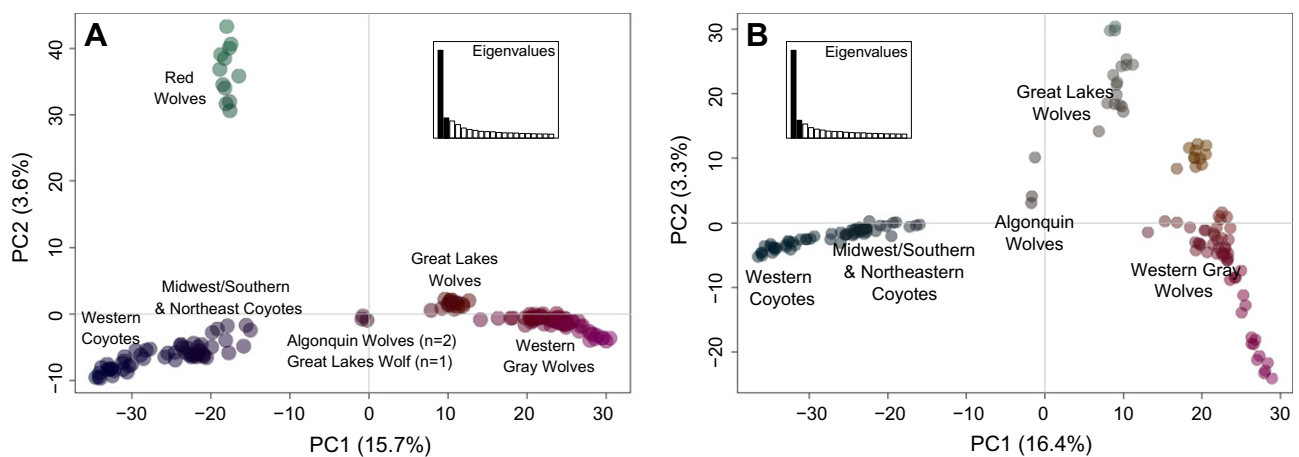


Fig. 1. Principal components analysis of 48 K SNP data. (A) Principal components analysis (PCA) (centered, unscaled) of 48 K SNP dataset (from vonHoldt et al., 2011) of 7 *Canis* populations. Dot color is based on the principal component values of individuals. (B) Principal components analysis (PCA) (centered, unscaled) of 48 K SNP dataset (from vonHoldt et al., 2011) of 6 *Canis* populations (excluding red wolves). Dot color is based on the principal component values of individuals. We had insufficient metadata to explain the "orange" cluster of "western gray wolves", but based on Fig. S2C in vonHoldt et al. (2011) they may be individuals from Northern Quebec and Ontario.

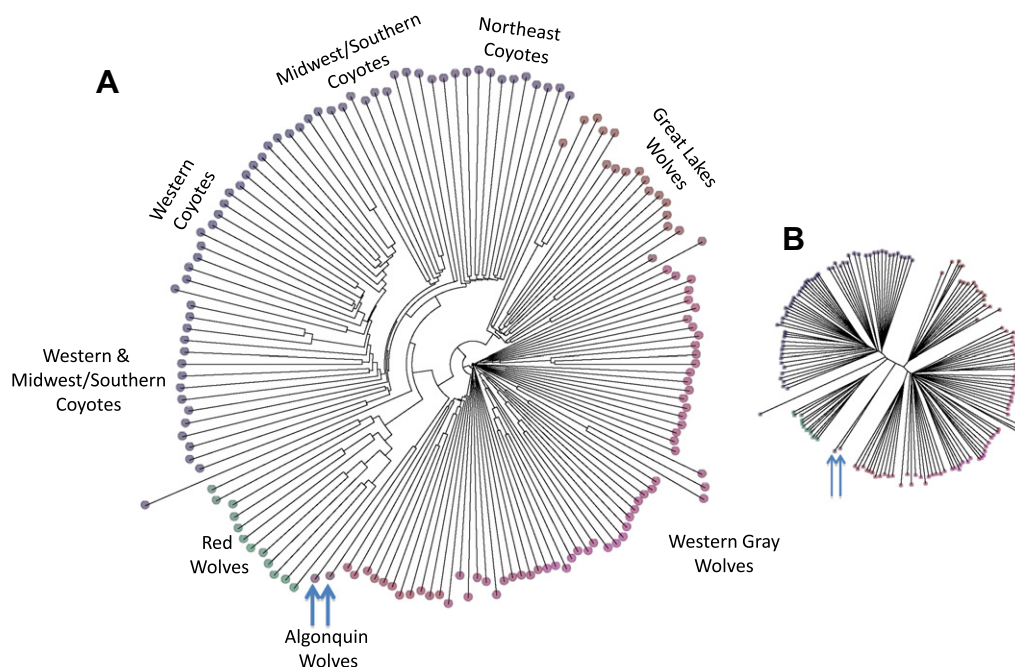


Fig. 2. Phylogenetic analysis of 48 K SNP data. (A) Phylogenetic tree constructed with the BIONJ algorithm for improved neighbor-joining in R. Dot color is based on the principal component values of individuals (as in Fig. 1A). (B) Unrooted version of phylogenetic tree. Blue arrows indicate Algonquin (i.e. eastern) wolves.

fully considered in the interpretation of the data presented by the authors. To provide further insight into the evolutionary history of the *Canis* genus, we performed novel analyses on the SNP data presented in vonHoldt et al. (2011). We conducted a centred, unscaled principal components analysis (PCA) implemented in the adegenet package (Jombart and Ahmed, 2011) of R 2.13.1 (R Development Core Team, 2011) of 48,036 SNPs of 154 individuals from 7 populations: Midwest Coyotes ($n = 19$), Western Coyotes ($n = 26$), Northeast Coyotes ($n = 13$), Western Gray Wolves ($n = 62$), Algonquin Wolves ($n = 2$), Great Lakes Wolves ($n = 20$), and Red Wolves ($n = 12$) (Fig. 1A). To test the consistency of methods utilized here and in vonHoldt et al. (2011), we conducted a PCA on the subset of coyote data (Midwest Coyotes, Western coyotes, and Northeast Coyotes) (see Supplementary Fig. S1). To visualize individual groupings, we used the colorplot function in R that assigns color variation based on the principal components values of each individual. The scale of our initial PCA was affected by the distinct clustering of red wolves, so we also used PCA to explore relative positioning of North American wolves and coyotes when red wolves were excluded from the analysis (Fig. 1B). To understand the phylogenetic relationship of the wolves from Algonquin Park to the other *Canis* types, we constructed a rooted (Fig. 2A) and unrooted (Fig. 2B) phylogenetic tree of the 48 K SNP dataset with the BIONJ algorithm (Gascuel, 1997) in R 2.13.1 (R Development Core Team, 2011). Individuals in the phylogenetic output are color-coded based on the scores assigned in the PCA of the same dataset (Fig. 1A). Finally, to elucidate relationships among the *Canis* types, we conducted a centred, unscaled PCA on a North American *Canis* dataset based on 12 previously published autosomal microsatellite markers (see Wheeldon et al., 2010b for genotyping methods) (accessed through the Wolf and Coyote DNA Bank at Trent University database (<http://wolf.nrdpfc.ca/>)).

3. Results and discussion

3.1. Sample selection and ancestry assignment

First, wolves from the Great Lakes region contain both gray wolf and eastern wolf genes (Wheeldon and White, 2009; Fain et al., 2010) but they are not representative of the historic eastern wolf that has been proposed as sharing a common evolutionary history in North America alongside coyotes (Wilson et al., 2000). Previous work on mitochondrial DNA (mtDNA) (Wilson et al., 2000; Grewal et al., 2004), Y-chromosome DNA (Wilson et al., in press), and microsatellite loci (Rutledge et al., 2010a) suggests that the wolves in Algonquin Provincial Park, Ontario, Canada are currently the best representative of the eastern wolf. Although hybridization with gray wolves and coyotes has been noted in *individuals* within the Algonquin Park population (Grewal et al., 2004; Wilson et al., 2009; Rutledge et al., 2010a), the influence from gray wolves is limited and the ongoing influence from coyotes has been curtailed by expanded protection from harvesting (Rutledge et al., 2011). It is also important to note that although coyote mtDNA is now relatively common in the Algonquin wolf population, mtDNA introgression can occur in the absence of nuclear introgression (e.g. Nevado et al., 2009). In analyses of autosomal microsatellites, wolves from Algonquin Provincial Park consistently cluster independent of other wolves from northeastern Ontario and the Great Lakes states, and separate from (but more closely to) eastern coyotes (Rutledge et al., 2010a; Wheeldon, 2009; Way et al., 2010). Therefore, from a nuclear genomic perspective, the Algonquin wolf population is likely to be the best representative of eastern wolves. Assuming a common origin for Great Lakes wolves and Algonquin Park wolves confounds the evolutionary reconstruction of both types of wolves and although they may both have an eastern wolf origin, they likely

have different historic and contemporary hybridization dynamics. Any test of the hypothesis that eastern wolves are a distinct species, *C. lycaon*, would require analyses of sufficient numbers of wolves from Algonquin Provincial Park that have been identified with a nuclear genetic signature consistent with an eastern wolf heritage. The approach of vonHoldt et al. (2011) to simply utilize the C1 mtDNA haplotype as an eastern wolf species diagnostic, is insufficient and inappropriate because hybridization between eastern wolves and coyotes has resulted in a widespread geographic range of the C1 haplotype throughout coyotes across eastern North America (Kays et al., 2010; Way et al., 2010; Wheeldon et al., 2010a). Although the authors include two Algonquin samples, there is no informative metadata (e.g. date sampled, weight, paternal heritage, etc.) associated with those samples and those two individuals could have been transient eastern coyotes or first generation hybrids between an Algonquin eastern wolf and a gray-eastern hybrid wolf from north-eastern Ontario, or an Algonquin eastern wolf-eastern coyote hybrid (Rutledge et al., 2010a). Although a principal components analysis (PCA) of the data with different subgroupings suggests the two Algonquin wolf samples are not eastern coyotes, the two potential hybrid scenarios remain unresolved (Fig. 1A). Similarly, in assessing the evolutionary connection between eastern wolves and red wolves, the comparison should be between the population of Algonquin wolves and red wolves (as done in Wilson et al., 2000), not Great Lakes wolves and red wolves (as done in vonHoldt et al., 2011). Again, this analysis would require sufficient sample sizes.

Second, if the purpose is to test the *C. lycaon* hypothesis, dogs should be excluded from the analysis because their long history of selective breeding skews the output from both STRUCTURE and the PCA. In the PCA presented in Fig. 3 of vonHoldt et al. (2011), 10% of the variation is explained by PC1 but only 1.7% explained by PC2. When the dog dataset is removed, however, in Supplemental Fig. S2 A–D of vonHoldt et al. (2011), the variation is more equally spread between the axes, and in a PCA of North American wolves (Supplemental Fig. S2-C of vonHoldt et al. (2011)), western wolves and Great Lakes wolves segregate along PC2 with the two Algonquin samples at the fringe of the Great Lakes wolf group. This clustering of the two Algonquin samples is more clearly demonstrated in a PCA of subgroups shown here in Fig. 1A and B. In the reanalysis, the two Algonquin samples appear intermediate to Great Lakes wolves and eastern coyotes, and red wolves are clearly distinct from the other groups (Fig. 1A). What remains unclear is *why* this pattern occurs. For the red wolves, one can speculate that a genetic bottleneck and subsequent drift occurred as a result of the captive breeding program that limited the gene pool to that of only 14 founding members (Hedrick and Fredrickson, 2008). However, the positioning of the Algonquin samples is more difficult to interpret, again due to the small sample size and lack of metadata. They are, however, clearly different (Fig. 1B), thus suggesting further sampling and investigation is warranted.

Similarly, in assessing the most likely number of clusters from the STRUCTURE analysis presented by vonHoldt et al. (2011), the inclusion of dogs skews the ΔK value for assessing the number of clusters in Supplemental Fig. S7A, but when only North American wolves are analyzed, ΔK identifies 4 main clusters. Those animals identified as “admixed canids” in Fig. 4 of vonHoldt et al. (2011), however, are not included in their optimal cluster analysis without dogs. Regardless, the inclusion of only 2 Algonquin wolf samples is insufficient to resolve a distinct eastern wolf lineage in STRUCTURE. Of interest, however, is the branching pattern of those 2 Algonquin samples in a phylogenetic analysis of the 48 K SNP data (Fig. 2). The phylogeny is concordant with the PCA (Fig. 1A) but the branching pattern suggests not only that the Algonquin wolves are very different from other *Canis* species, but that they are closely related to red wolves and more importantly are basal to the other North American lineages (Fig. 2A). This pattern is also observed

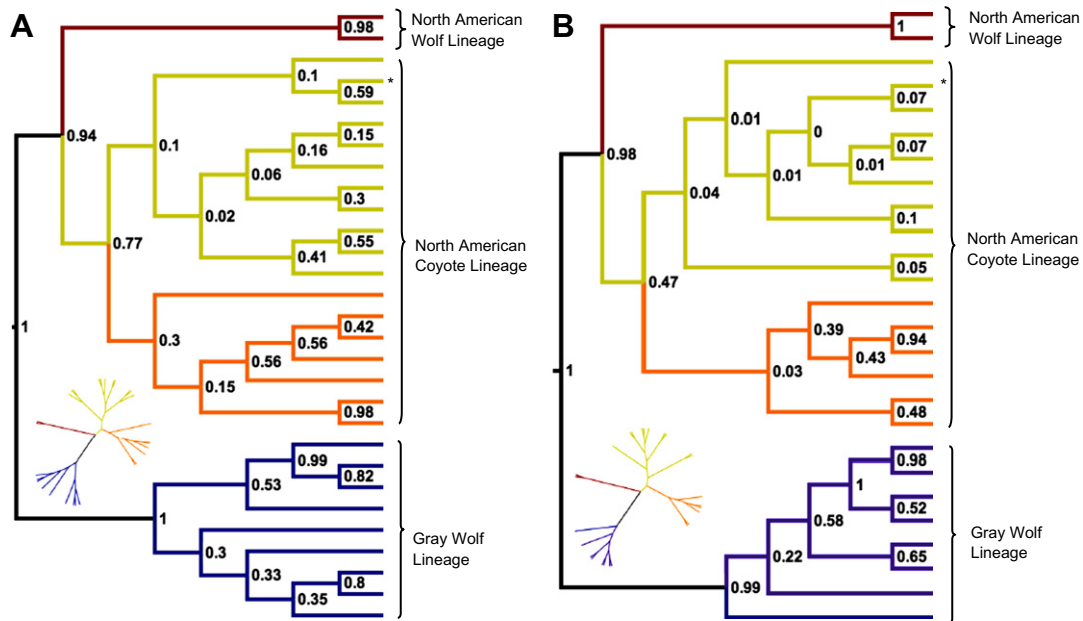


Fig. 3. Phylogenetic analysis of *Canis* mtDNA. (A) Phylogenetic analysis of *Canis* mitochondrial DNA control region. (B) Phylogenetic analysis of *Canis* mitochondrial DNA ATPase genes. Fig. adapted from Rutledge et al., 2010b with permission. Branch labels are posterior probabilities. * indicates the putative red wolf mtDNA haplotype.

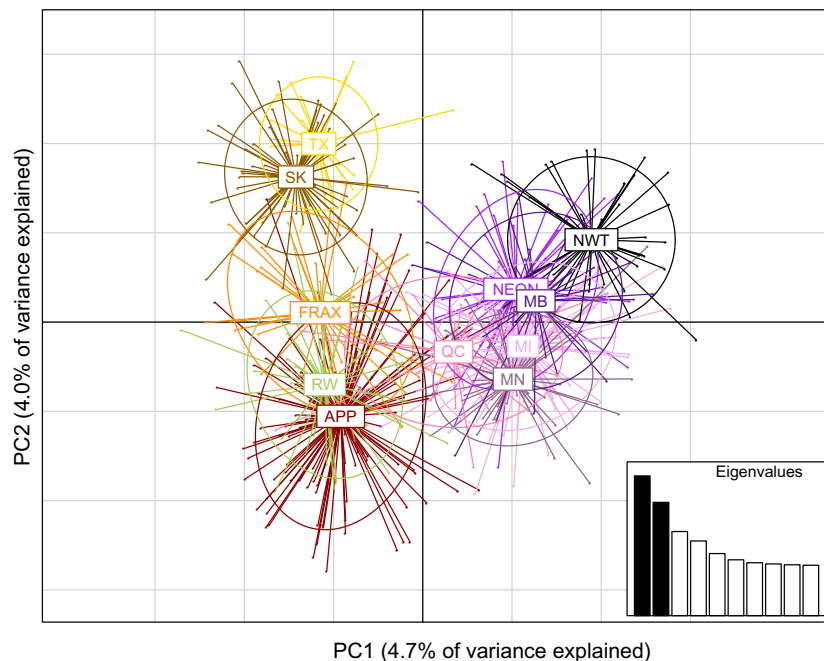


Fig. 4. Centred, unscaled PCA of North American wolves and coyotes based on 12 microsatellites. APP (Algonquin Provincial Park; $n = 125$), RW (Red Wolves – Captive Breeding Program; $n = 44$), FRAX (Frontenac axis; $n = 38$), SK (Saskatchewan; $n = 68$), TX (Texas; $n = 23$), NWT (Northwest Territories; $n = 43$), NEON (Northeast Ontario; $n = 51$), MB (Manitoba; $n = 33$), MI (Michigan; $n = 83$), MN (Minnesota; $n = 50$), QC (Quebec; $n = 28$). Clear delineation along PC1 between Old World evolved wolves (NWT/ Great Lakes-Boreal regions) and New World evolved wolves (APP, RW), eastern coyotes (FRAX), and western coyotes (TX, SK).

in phylogenies based on coding and non-coding regions of the mitochondrial DNA (Rutledge et al., 2010b; Fig. 3). Although we hesitate to make hard conclusions about the analysis based on only 2 samples, the phylogenetic pattern observed in the SNP data clearly warrants further investigation with a larger sample size.

Finally, the ancestry analysis shown in Figure 6 of vonHoldt et al. (2011) assumes a two-species model with the western coyote and western gray wolf set as the ancestral reference populations (see Supplemental methods of vonHoldt et al., 2011). This does not, therefore, contribute to the test of a *C. lycaon* hypothesis be-

cause the admixture can only be categorized between the two species as systematically defined prior to the analysis. Different model assumptions (e.g. a three-species model) would, therefore, lead to different outcomes.

3.2. SNPs in isolation of other genetic markers

Although large numbers of SNPs can be a very effective tool for identifying genetic structure in wild populations (see Helyar et al., 2011 and references therein), inferences based on SNP data should

not be made in isolation of other genetic markers that are informative on different evolutionary time scales and/or based on different patterns of inheritance. For example, the higher allelic diversity per locus and higher mutation rate of microsatellites make them an important marker for identifying recent divergence. As shown here in Fig. 4, a PCA of autosomal microsatellite genotypes provides a clear separation along the first principal component of North American evolved *Canis* and Eurasian evolved *Canis* species, whereas separation along PC2 partitions the North American lineage into wolves (Algonquin (i.e. eastern) wolves/red wolves) and coyotes (Texas and Saskatchewan), with eastern coyotes (Frontenac Axis in southern Ontario) intermediate to the other two. Similarly, the Eurasian evolved lineages are separated (although less dramatically) along PC2 with gray wolves from the Northwest Territories set apart along a gradient from those in Manitoba, North-eastern Ontario, and the Great Lakes states. Individuals from Quebec represent a mixture of canid types (both coyotes and wolves). The PCA also suggests some individuals sampled within specific locations are more heavily influenced by hybridization than others because they fall within other clusters, but the confidence ellipses (with the exception of Quebec) do not overlap across PC1, nor do they intersect between North American evolved wolves (APP/RW) and western coyotes (SK/TX) along PC2. The microsatellite PCA gives a broad perspective on a three-species model of North American *Canis* evolution within the context of hybridization events.

Within hybridized populations, maternal or paternal markers examined on their own are insufficient for species assignment. However, markers associated with mtDNA and the Y-chromosome contribute to our understanding of evolutionary lineages in a historic context. Both the mtDNA and Y-chromosome show distinct eastern wolf lineages that support the conclusion of an historic North American wolf that evolved independent of the gray wolf (Rutledge et al., 2010a,b; Fain et al., 2010; Wilson et al., in press). The complete evolutionary history of the eastern wolf becomes obscured when examining genetic variation across autosomal DNA alone due to *Canis* hybridization throughout eastern North America. In addition, the limitations of traditional F_{ST} estimates applied to SNP data (see Helyar et al., 2011) and the potential for biased clustering analysis due to the effect of variation in neutrality of SNP loci (Satkoski Trask et al., 2011) need to be considered.

Ascertainment bias is also another common problem with SNP data. Although the authors attempt to address ascertainment bias associated with SNPs identified primarily in the boxer and poodle genomes to make inferences about wild ancestors, we are not convinced that the bias is sufficiently negated. Selective breeding in dogs over the past 15,000 years is likely to have resulted in differential variation across the genome compared to wild relatives. SNP detection from a limited discovery panel is known to over-represent high frequency alleles (Nielsen et al., 2004) and bias comparisons of genetic variability (Clark et al., 2005). It has been noted that SNP discovery samples sizes less than 3 can cause bias against rare alleles and 10 individuals has been suggested as a minimum standard to provide unbiased genetic diversity (Satkoski Trask et al., 2011). Given the variation in wild canids analyzed, it is unlikely that their genetic variation would be adequately captured by the domestic dog SNP array used, especially because ascertainment bias associated with SNP detection from the dog genome has proven problematic in other studies on wolf populations (Seddon et al., 2005). We predict that SNPs ascertained from a non-domestic *Canis* source would capture more genetic variation and provide further delineation of the eastern wolves from Algonquin Park beyond that presented here.

3.3. Genetics in isolation of ecology, natural history, and the fossil record

The conclusion that eastern wolves (called “Great Lakes wolves” by vonHoldt et al., (2011)) are the result of gray wolf and coyote hybridization ignores ecological data. Mech (2012) reports on three main non-genetic reasons that support a distinct eastern wolf lineage in eastern North America. First, eastern wolves are morphologically intermediate in size between gray wolves and coyotes (Kolenosky and Standfield, 1975; Schmitz and Kolenosky, 1985) with no evidence of similarly intermediate sized wolves in western North America.

Second, early reproductive experiments demonstrated that a 25 kg female “gray wolf” (*C. lupus lycaon*) and a 14 kg male coyote (*C. latrans thomsoni*) produced viable offspring in captivity (Kolenosky, 1971). However, the female wolf was captured from Algonquin Provincial Park and the male coyote was taken from a den 48 km north of Toronto. This interbreeding was therefore likely between an animal with predominantly eastern wolf (*C. lycaon*) ancestry and an eastern coyote (*C. latrans x lycaon*), which readily interbreed in the wild (Rutledge et al., 2010a), not between a western gray wolf (*C. lupus*) and a western coyote (*C. latrans*), for which there is no evidence of interbreeding neither in the wild where they occur sympatrically nor in captivity.

Third, in western North America, wolves often kill transient coyotes (Berger and Gese, 2007), whereas the same interaction is not observed east of Michigan (Mech, 2012). The intolerant behavior observed between western gray wolves and western coyotes makes natural interbreeding between the two species highly improbable and such a mating has never been documented. While some behavioral interactions have been noted between gray wolf hybrids and coyote hybrids in the Great Lakes region (Thiel, 2006), genetic evidence demonstrates that interbreeding between them does not typically occur (Wheeldon et al., 2010b; Fain et al., 2010), thereby supporting the idea that coyote-like mtDNA found in Great Lakes wolves is of eastern wolf origin. Hybridization is prevalent in the east because the intermediately sized eastern wolf bridged gene flow between two species that could not directly interbreed (Wheeldon and White, 2009; Rutledge et al., 2010a).

Fourth, the presence of two wolf types in eastern North America is recorded in historical accounts. In *The Origin of Species*, Darwin (1859) notes that “... there are two varieties of the wolf inhabiting the Catskill Mountains in the United States...”, and almost 200 years prior to that in *New England's Rarities Discovered*, Josselyn (1672) described two different sized wolves in Massachusetts: “The wolf, of which there are two kinds; one with a round ball'd foot, and are in shape like mongrel Mastiffs; the other with a flat foot, these are liker Greyhounds, and are called *Deer Wolves*, because they are accustomed to prey upon deer.” Similarly, DeKay (1842) noted in *The Natural History of New York* that the state had two types of wolves which he called a gray wolf that had “short reddish hairs” in summer and a very rare black wolf that was “more bulky and powerful” than the other type.

Finally, the fossil record documents that *C. lupus* was not present in North America until the Illinoian period (~300,000 ybp) (Nowak, 1979) or perhaps late Rancholabrean period (~130,000–10,000 ybp), with little evidence that *C. lupus* occurred south of the glaciers prior to the late Rancholabrean (Nowak, 2002). Although large wolves (*C. dirus*) and coyotes (*C. latrans*) were originally present in the east, they disappeared at the end of the Pleistocene (~11,000 ybp), whereas a smaller wolf (distinct from western *C. lupus* and presumably *C. lycaon/C. rufus*) persisted until extirpation began in conjunction with the arrival of European settlers approximately 500 ybp (Nowak, 2002). Thus, the fossil record

supports the evolution of smaller North American wolf that evolved independent of the gray wolf in the regions east of the Mississippi River.

4. Conclusions

Based on the genetic data in combination with a broader scientific perspective, the *C. lycaon* hypothesis cannot be rejected. We conclude, therefore, that the biases introduced by vonHoldt et al. (2011) through the methodological design of the species datasets grouped for analyses (i.e. gray wolves and coyotes), along with the limited sample size of representative eastern wolves from Algonquin Provincial Park, and the unresolved issues associated with SNP analyses and ascertainment bias, render the conclusion of a two-species model for North American canid evolution tenuous at best. Limitations of the SNP dataset need to be acknowledged and data from different types of genetic markers, morphology, behavior, and the fossil record need to be taken into account before a three-species evolutionary hypothesis can be rejected. Thus, the conclusions of vonHoldt et al. (2011) need to be tempered and their interpretation reconsidered, especially given the severity of the consequences associated with making a type II error (i.e. incorrectly rejecting the *C. lycaon* hypothesis) as it relates to endangered species legislation in Canada and the United States. Overall, this report provides a cautionary tale for the burgeoning field of conservation genomics of interpreting genomic data from non-representative samples to the exclusion of all other available biological information. As the field of conservation genetics evolves into conservation genomics, with all its exciting opportunities, researchers must not forget the equally important role of interdisciplinary research to understand the biology of endangered species. Failure to do so could lead to severe repercussions for wildlife if misinformed conservation policies are implemented.

Note

Though not important for the purposes of this commentary, a correction to the text of vonHoldt et al. (2011) is that the reference to samples from the 400 to 500-year-old site described in Rutledge et al. (2010c) are from southern Ontario, not from Quebec.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocon.2012.05.017>.

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