

Spatial genetic and morphologic structure of wolves and coyotes in relation to environmental heterogeneity in a *Canis* hybrid zone

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Abstract

Eastern wolves have hybridized extensively with coyotes and gray wolves and are listed as a 'species of special concern' in Canada. However, a distinct population of eastern wolves has been identified in Algonquin Provincial Park (APP) in Ontario. Previous studies of the diverse *Canis* hybrid zone adjacent to APP have not linked genetic analysis with field data to investigate genotype-specific morphology or determine how resident animals of different ancestry are distributed across the landscape in relation to heterogeneous environmental conditions. Accordingly, we studied resident wolves and coyotes in and adjacent to APP to identify distinct *Canis* types, clarify the extent of the APP eastern wolf population beyond the park boundaries and investigate fine-scale spatial genetic structure and landscape-genotype associations in the hybrid zone. We documented three genetically distinct *Canis* types within the APP region that also differed morphologically, corresponding to putative gray wolves, eastern wolves and coyotes. We also documented a substantial number of hybrid individuals (36%) that were admixed between 2 or 3 of the *Canis* types. Breeding eastern wolves were less common outside of APP, but occurred in some unprotected areas where they were sympatric with a diverse combination of coyotes, gray wolves and hybrids. We found significant spatial genetic structure and identified a steep cline extending west from APP where the dominant genotype shifted abruptly from eastern wolves to coyotes and hybrids. The genotypic pattern to the south and northwest was a more complex mosaic of alternating genotypes. We modelled genetic ancestry in response to prey availability and human disturbance and found that individuals with greater wolf ancestry occupied areas of higher moose density and fewer roads. Our results clarify the structure of the *Canis* hybrid zone adjacent to APP and provide unique insight into environmental conditions influencing hybridization dynamics between wolves and coyotes.

Keywords: cline, coyotes, eastern wolves, generalized additive mixed models, hybrid zone, hybridization

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Introduction

Identifying the spatial distribution of genotypes and phenotypes across hybrid zones has long been a goal of evolutionary ecologists seeking to infer the processes

generating and maintaining hybrid zones (Mayr 1963; Endler 1977; Barton & Hewitt 1985). Hybrid zones may be spatially structured as clines, where genotypes and phenotypes transition along a gradient from one parental type to the other (Barton & Hewitt 1985; Rand & Harrison 1989). Alternatively, hybrid zones may be mosaic in structure, where a patchwork of alternating genotypes and phenotypes is distributed across the

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landscape, usually in relation to environmental heterogeneity (Rand & Harrison 1989; Britch *et al.* 2001). Most studies of hybrid zones have sought to provide theoretical insight into evolutionary processes such as speciation (Mayr 1963; Endler 1977; Barton & Hewitt 1985). However, increasing recognition of the practical implications of hybridization, both negative (e.g. reduction of rare species, Rhymer & Simberloff 1996; Allendorf *et al.* 2001) and positive (e.g. rapid adaptation to new environments, Seehausen 2004; Mallet 2005), means that understanding the structure of hybrid zones can also be an important conservation objective. Specifically, understanding spatial variation of rare genotypes and identifying environmental conditions underlying these patterns are important goals for developing sound management strategies for hybridizing species.

The colonization of north-eastern North America by coyotes (*Canis latrans*) during the twentieth century led to widespread hybridization between coyotes and eastern wolves (*Canis lycaon*; Wilson *et al.* 2000; Kyle *et al.* 2006). This colonization was facilitated by human actions as forest-clearing and direct persecution reduced and eliminated wolves (*Canis* spp.) from much of the United States and southern Canada (Fritts *et al.* 2003) and may have also reduced reproductive barriers between wolves and coyotes (Kolenosky & Standfield 1975; Kyle *et al.* 2006). Eastern wolves also appear to have hybridized extensively with gray wolves (*C. lupus*) in the western Great Lakes Region and central Ontario (Fain *et al.* 2010; Wheeldon *et al.* 2010). Although considerable evidence suggests eastern wolves are a distinct species (e.g. Wilson *et al.* 2000; Kyle *et al.* 2006; Fain *et al.* 2010; Mech 2011), this designation remains controversial and an alternative viewpoint suggests intermediate sized wolves in eastern North America are the product of hybridization between gray wolves and coyotes (e.g. von Holdt *et al.* 2011). Eastern wolves are currently considered a subspecies of the gray wolf (*C. l. lycaon*) and are listed as a 'species of special concern' federally in Canada (COSEWIC 2001) and in the province of Ontario (COSSARO 2004). Regardless of uncertainty regarding their evolutionary history and distribution, eastern wolves are protected under Federal and Provincial Species at Risk Acts and a genetically distinct population of eastern wolves has been identified in Algonquin Provincial Park (APP) in Ontario (Rutledge *et al.* 2010).

A hybrid swarm has apparently replaced eastern wolves from many areas across Ontario such that few, if any, nonadmixed individuals remain (Wilson *et al.* 2009; Rutledge *et al.* 2010). However, most breeding wolves in APP are 'Algonquin-type' eastern wolves and genetically distinct from both eastern coyotes in south-eastern Ontario and admixed gray wolves (*C. lupus* ×

lycaon) in north-eastern Ontario (Rutledge *et al.* 2010). Although the APP population has been studied extensively within the park boundaries and compared with other populations across Ontario and beyond (Grewal *et al.* 2004; Rutledge *et al.* 2010), the full extent of the Algonquin-type eastern wolf population remains unknown as the spatial genetic and morphologic structure of the hybrid zones in many areas immediately adjacent to the park have not been well studied. The conservation status of eastern wolves in Canada is being reviewed by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) during 2012–2013; therefore, it is important to identify the spatial distribution of *Canis* genotypes in areas adjacent to APP.

Across the broad *Canis* hybrid zone in north-eastern North America, many studies have analysed molecular data and made inferences regarding the genetic ancestry of individuals in wolf and coyote populations (e.g. Wilson *et al.* 2000; Grewal *et al.* 2004; Koblmüller *et al.* 2009; Fain *et al.* 2010; Rutledge *et al.* 2010; Wheeldon *et al.* 2010; von Holdt *et al.* 2011). Previous researchers have speculated that observed variation in genetic structure of *Canis* populations could be related to interactions between canid body size, prey availability and genotype-specific responses to human disturbance (e.g. Wilson *et al.* 2000; Kyle *et al.* 2006; Koblmüller *et al.* 2009; Rutledge *et al.* 2010; von Holdt *et al.* 2011). However, no previous studies have extended their molecular results by showing that the observed genetic distinctions manifested in morphological or ecological differences across hybridizing *Canis* types. Rutledge *et al.* (2010) presented weights of animals from three regions of Ontario (including APP) inhabited by wolves and/or coyotes, but no genotype-specific analysis of morphology was conducted. Sears *et al.* (2003) conducted a detailed comparison of morphological characteristics of putative wolves, coyotes and hybrids in areas within and adjacent to APP; however, they did not provide genetic profiles of these animals. Sears *et al.* (2003) also compared *Canis* diet from scat analysis and landscape attributes across study sites, but did not connect these data to individual animals or genetic ancestry. Thus, studies explicitly linking genetic inferences with morphological and ecological characteristics of individual animals are clearly needed to begin to elucidate the biological significance of wolf-coyote hybridization.

Accordingly, we studied genetics, morphology and landscape associations of resident wolves and coyotes in and adjacent to APP with three main objectives and several associated questions and hypotheses. Our first objective was to characterize the genetic structure of *Canis* populations in the hybrid zone in and adjacent to

APP to: (i) identify distinct *Canis* genetic types, (ii) determine the extent of admixture between distinct types and (iii) investigate fine-scale spatial genetic structure. Specifically, we addressed the question of whether the hybrid zone adjacent to APP is structured as a cline or a mosaic and whether the pattern varies across the region. Second, we hypothesized that variable environmental conditions related to prey availability, habitat fragmentation and human disturbance would explain much of the variation in the distribution of wolf and coyote genotypes in and adjacent to APP. We predicted that wolves would be associated with areas of higher densities of large ungulates, whereas coyotes would be associated with areas of greater human disturbance. Third, we compared morphology of wolves, coyotes and admixed individuals to determine whether distinct genetic types also differed phenotypically. Specifically, we hypothesized that: (i) body size increases along a gradient from coyotes to eastern wolves to gray wolves, with distinct types exhibiting genotype-specific morphology and (ii) admixed individuals exhibit morphology intermediate to parental types. Our results will provide unique insight into the influence of a large protected area and variable environmental conditions in adjacent areas, on the structure of a hybrid zone between three putative *Canis* species.

Materials and methods

Study area

We studied wolves and coyotes in central Ontario in four study units within our approximately 26 800 km² study area in and around Algonquin Provincial Park (APP): (i) western APP (APP), (ii) Wildlife Management Unit 49 (WMU49), (iii) Kawartha Highlands (KH) and (iv) Wildlife Management Unit 47 (WMU47; Fig. 1). Although we documented dispersal of nonresident animals between these study units, there was no overlap between the four study units in terms of the home ranges of resident animals (home range estimation explained in Appendix S1, Supporting information). In APP, we monitored animals mostly in the western portion of the 7715 km² Provincial Park (Fig. 1). Except for small portions of the home ranges of two packs, the ranges of APP animals were within the park and the surrounding buffer zone (park + buffer area = 15 623 km²) within which wolf and coyote harvest has been banned since December 2001 (Fig. 1). Wolf and coyote harvest by trapping and hunting was allowed, on a seasonal or year-round basis, in the three study units outside of APP with the exception of smaller protected areas within KH. Specifically, wolf and coyote hunting was illegal (but trapping was allowed) in Kawartha

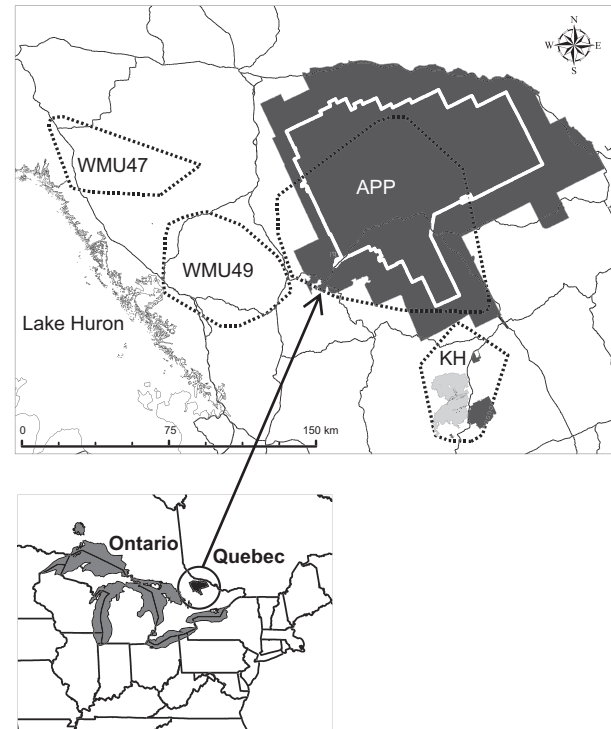


Fig. 1 The four study units (APP, WMU47, WMU49, KH) in central Ontario denoted by minimum convex polygons (dashed outlines) created using telemetry data from study animals. Dark grey shading represents areas where wolves and coyotes were protected from harvest, whereas light grey shading indicates trapping (but no hunting) was allowed. White polygon shows the APP boundary, and black lines represent major roads.

Highlands Signature Site (KHSS, 375 km²), whereas both hunting and trapping were banned in the Peterborough Crown Game Reserve (152 km²) and in four smaller (<16 km²) areas in KH (Fig. 1). However, no resident wolves and coyotes we monitored in KH were provided complete protection from harvest, as home ranges of all study animals in areas adjacent to APP extended into areas where harvest was legal.

Canis types

Given the taxonomic uncertainty surrounding some *Canis* species and populations, it is important (but challenging) to use clear and consistent terminology when discussing wolves and coyotes in eastern North America (Cronin & Mech 2009). Hereafter, we refer to Algonquin-type eastern wolves (Rutledge *et al.* 2010) as eastern wolves. Admixed gray wolves in the western Great Lakes Region and Ontario have experienced contemporary and/or historical hybridization (Kobl Müller *et al.* 2009; Fain *et al.* 2010; Wheeldon *et al.* 2010; von Holdt *et al.* 2011), and we refer to them as gray wolves

for simplicity. Eastern coyotes (hereafter coyotes) in the APP region cluster with south-eastern Ontario coyotes in population genetic analyses (J. Benson & B. Patterson, unpublished data) and have a history of hybridization with eastern wolves (Rutledge *et al.* 2010; Way *et al.* 2010). Thus, although we refer to animals in our study area as eastern wolves, gray wolves and coyotes for simplicity, we do not suggest that the animals we studied are 'pure' representations of the ancestral genomes of these taxa, nor do our questions and inferences require acceptance of a specific evolutionary model. Rather, we acknowledge varying and uncertain levels of recent and historical gene flow between *Canis* populations in Ontario and seek to provide insight into whether canids presently inhabiting the APP region are genetically and morphologically distinct, spatially structured and associated with specific environmental conditions. Consistent with the recommendation of Cronin & Mech (2009), we believe that maintaining fit wolf populations is an important management goal and that more research should be directed towards understanding their ecological and demographic status.

Sample collection and field methods

We obtained DNA samples from 342 wolves and coyotes mostly from live capture ($n = 272$) using padded foothold traps, helicopter net-gunning, modified neck snares and capture by hand (neo-natal pups only). We weighed and measured captured animals, recording body mass (kg) and body length (cm; tip of nose to base of tail). We captured animals in APP during 2006–2011, WMU49 during 2006–2011, KH during 2009–2010 and WMU47 during 2004–2005. Blood was taken from the cephalic vein and deposited on FTA cards (Whatman Ltd) that were stored at room temperature until processing. We collected noninvasive hair and scat (swabbed for DNA in the field, Rutledge *et al.* 2009) samples from kill, den and rest sites of focal packs and opportunistically while conducting field activities. We also collected tissue samples from road-killed animals. After processing, we compared successful noninvasive samples with those from captured animals to identify matching genotypes and family relationships. We identified 70 unique genotypes from noninvasive sampling beyond those that matched previously genotyped animals. Additional field methods are described in Appendix S1, Supporting information.

Microsatellite genotyping

We amplified 12 autosomal microsatellite loci for each sample (Ostrander *et al.* 1993, 1995; cxx225, cxx2, cxx123, cxx377, cxx250, cxx204, cxx172, cxx109, cxx253,

cx442, cxx410, cxx147) as in Wheeldon *et al.* (2010). Genotyping was performed on an ABI3730 (ABI, Applied Biosystems), and alleles were scored in GeneMarker v1.7 (Softgenetics LLC). We quantified noninvasive (i.e. low template) samples based on nuclear DNA (at locus cxx204) to ensure that ≥ 250 –500 pg/ μ L of DNA was available before proceeding with microsatellite profiling. For noninvasive hair and scat samples, if extraction yielded <250 pg/ μ L, we did not attempt microsatellite amplification. We re-submitted PCR product at lower dilution and/or re-amplified samples with low amplifying alleles to confirm homozygous genotypes or if there was any ambiguity in scoring alleles. We also amplified a 343–347-bp fragment of the mitochondrial DNA control region (for all individuals) and four Y-chromosome microsatellite loci (for males, MS34A, MS34B, MS41A, MS41B; Sundqvist *et al.* 2001) for use in parentage analysis (details in Appendix S1, Supporting information). All laboratory processing of DNA samples, from extraction to scoring, was carried out in a single laboratory, on the same equipment, and with consistent protocols. Additional details of laboratory methods are available in Wheeldon *et al.* (2010).

Sample information

We included microsatellite profiles from 121 and 146 individuals from the four study units in analyses assessing genetic structure for: (i) residents only and (ii) residents and transients, respectively. We also included samples from 40 north-eastern Ontario (NEON) gray wolves as an outgroup ($n = 40$, Rutledge *et al.* 2010), because we suspected admixed gray wolves existed in our data set. Thus, for our main analysis, we included samples from 161 total individuals from APP ($n = 40$), WMU49 ($n = 42$), KH ($n = 21$), WMU47 ($n = 18$) and NEON ($n = 40$). Sample types included blood ($n = 144$), scat ($n = 5$), hair ($n = 4$) and tissue ($n = 1$). We also reconstructed genotypes from seven breeding individuals for inclusion in our analyses based on genotypes of one known breeder (identified with pedigree analysis, refer to Appendix S1, Supporting information) and ≥ 4 known offspring from a single litter. We reconstructed these genotypes using the alleles of the known breeder and those of the pups following principals of Mendelian inheritance implemented in the program Gerud 2.0 (Jones 2005). We limited our main analysis to resident pack animals ($n \geq 57$ packs) because we were interested in assessing genetic structure of the resident, breeding units of wolves and coyotes across the study area. Based on radio-telemetry data, we excluded non-resident animals ($n = 25$) that were solitary (not with a pack) and did not exhibit home range behaviour. We

monitored reproductive status of all radio-collared females in our study each spring using telemetry to identify natal den sites, but did not document reproduction by nonresidents. Thus, our findings were similar to other studies that have indicated that breeding by nonresident wolves is extremely rare (Mech & Boitani 2003). We provide further details regarding our determination of residency status for all animals in Appendix S1, Supporting information.

We excluded all direct offspring of breeding animals from our analyses ($n = 144$), unless we did not have samples for both breeding animals in a given pack (see below). Most (78%) of these offspring were captured as neonatal (3–6.5 weeks old) pups in natal dens as part of a companion study of pup survival. When both parents were included in the analysis, we also excluded offspring identified by our parentage analysis from samples obtained by other captures (i.e. not at den sites, 19%) or by noninvasive sampling (3%). We excluded these offspring to avoid unstable and potentially spurious results from population genetic analyses, which can arise because relatedness among family members may be difficult to distinguish from population structure (Camus-Kulandaivelu *et al.* 2007). Given that we sampled offspring unequally from our focal packs (range 0–15 pups per pack), including all offspring would have biased our analyses by over-representing the genotypes of packs sampled more intensively. In many cases, we had both breeding animals genotyped from our focal packs ($n = 60$ breeders from 30 packs) and both were included in our analyses. When we had 0 or 1 breeder genotyped, and reconstruction of the other breeder was not possible, we included two offspring ($n = 5$) or a breeder and an offspring ($n = 3$), respectively, to represent the parental genotypes. In other cases ($n = 13$), we included a single adult from a pack. We also included any resident individuals ($n = 2$) that were unrelated (not direct offspring or siblings) to the breeding animals in our focal packs. The only instances in which we included direct offspring of breeding pairs in our analyses were cases where these offspring joined or formed other packs later in the study, or became breeding animals themselves within their natal packs. Finally, we did not include noninvasive samples ($n = 55$) if we were unable to link them to our focal packs via pedigree analysis and/or field data. This was because we had no way of knowing whether these animals were residents or nonresidents and could have simply been dispersing through the area. The exclusions noted above decreased our overall sample size, but strengthened our inferences by ensuring that our results were unbiased and directly relevant to the resident, breeding *Canis* population in our study area.

Genetic structure analyses

We obtained autosomal microsatellite genotypes based on 10 ($n = 1$), 11 ($n = 2$) or 12 ($n = 158$) loci for our main analysis. We analysed autosomal genotype data in several ways to assess population structure and investigate sources of genetic variation in wolves and coyotes across our study area. First, we used a Bayesian approach, implemented in the program Structure (v.2.3.3, Pritchard *et al.* 2000) to identify genetic clusters and to estimate genetic origin of individuals using microsatellite allele frequencies. The Structure analysis allows for estimation of admixture proportion (Q) that is an estimate of the proportion of an individual's genome derived from a given genetic population (Falush *et al.* 2003). We ran the admixture model of Structure, assuming correlated allele frequencies and inferring the parameter alpha, for $K = 1$ –7 with five repetitions of 10^6 iterations following a burn-in period of 250 000 iterations for each K . We calculated the posterior probability ($\ln P[D]$) for each K by averaging $\ln P[D]$ across the five runs. We evaluated relative support for each value of K based on the mean $\ln P[D]$ (Pritchard *et al.* 2000) and ΔK (Evanno *et al.* 2005), and we also considered the biological significance of each potential number of clusters. We conducted a second Structure analysis in which we included nonresident animals ($n = 25$) and replaced one pup with their mother or father for packs ($n = 4$) where we only had one breeder sampled and had included 1 or 2 pups to represent genotypes of the breeding animals. We conducted this second analysis to obtain individual assignments for all breeding and nonresident animals not included in the main analysis for use in subsequent analyses.

Next, we conducted a centred principal components analysis (PCA) with the autosomal microsatellite allele data using the 'ade4' package v. 1.3-1 (Jombart 2008) in R v. 2.13.1 (R Development Core Team 2011) to corroborate inferences from the Structure analysis by arranging individuals in our study area along axes of variation based on their microsatellite allele genotypes (Patterson *et al.* 2006). After running the PCA, we calculated the percentage of the total variance explained by each component and calculated 95% confidence ellipses for groups of individuals, organized by study unit of residency, to assess the genotypic composition of each study unit. PCA is also an effective dimension reducing method to prepare microsatellite data for an alternative clustering procedure, K -means, which partitions genetic variation into between-group and within-group components and attempts to minimize the latter to find cohesive clusters (Lee *et al.* 2009; Jombart *et al.* 2010). K -means, when used with Bayesian Information Criteria (BIC) to determine the best supported model, has been

shown to perform similarly or better than Structure (with $\text{LnP}[D]$ and ΔK) in terms of determining the number of clusters in genetic data (Liu & Zhao 2006; Lee *et al.* 2009; Jombart *et al.* 2010).

Individual assignments

We repeated the Structure procedure at the highest, strongly supported value of K for 10 repetitions and averaged Q -values across the 10 runs for use with individual assignments. We classified individuals of $q \geq 0.8$ as belonging to a specific cluster and individuals with all $q < 0.8$ as being admixed, consistent with the previous *Canis* research (Verardi *et al.* 2006; Rutledge *et al.* 2010; Wheeldon *et al.* 2010). Although analysis of microsatellite data with program Structure has been recommended for individual assignment and detecting hybrids (e.g. Manel *et al.* 2005; Vähä & Primmer 2006), we verified the assignments using PCA. We employed this additional step because of the inherent difficulty of detecting backcrossed hybrids between closely related species with a recent history of admixture (Randi 2008) and because PCA does not require genetic assumptions that, if violated, can compromise accuracy of individual assignment in Structure (Paschou *et al.* 2007). Furthermore, we recognize the arbitrary nature of q -value thresholds for determining hybrid status (Vähä & Primmer 2006), making it important to verify our assignments with additional analysis subsequent to the application of our threshold criteria. Thus, after placing individuals into genotype classes, we used PCA to calculate 95% confidence ellipses with individuals grouped by their assigned cluster, to evaluate whether the original assignments agreed with the clustering of individuals along axes of variation in the PCA. If the PCA indicated that an individual was within, intersecting or beyond the 95% confidence ellipse of another group (highly assigned or admixed groups), we assumed that individual belonged in that cluster. Additionally, we used PCA to clarify ancestry of four individuals whose Q scores suggested possible admixture between >2 clusters. Our approach follows Cegelski *et al.* (2003) and Bohling & Waits (2011) by using multiple analytical approaches to improve confidence in individual genetic assignments, which is particularly important for studies with management implications.

Spatial genetic structure

We conducted a spatial principal components analysis (sPCA) to investigate spatial genetic patterns among wolves and coyotes in and adjacent to APP and to identify areas in this landscape where eastern wolves persist. sPCA utilizes Moran's I , an index of spatial

autocorrelation, to compare allele frequencies observed in individuals at given spatial locations with those of individuals at neighbouring sites (Jombart *et al.* 2008). Jombart *et al.* (2008) developed two multivariate tests for use with sPCA to detect global (e.g. clines and patches) and local structure. Significant global structure is identified when individuals that are spatially close are also similar genetically (positive spatial autocorrelation), whereas significant local structure is identified when individuals that are spatially close are dissimilar genetically (negative spatial autocorrelation). For spatial locations, we used the centroid of the home range for all animals in packs with sufficient GPS telemetry data ($n = 95$). For other animals, we used the mean centre of all telemetry locations ($n = 15$), capture location ($n = 6$), sample location (for noninvasive samples, $n = 4$) or den site location ($n = 1$). To facilitate this analysis, we developed a Gabriel's graph (Legendre & Legendre 1998) as a connection network to model the spatial relationships between individuals. In contrast to the previous analyses (Structure, PCA, K -means), we did not include the NEON out-group because we were interested in investigating spatial genetic relationships only within our study area.

We used generalized additive mixed models (GAMMs; Lin & Zhang 1999; Wood 2006) implemented with the packages 'gamm4' v. 0.1-6, 'mgcv' v. 1.7-6 and 'lme4' in R to further investigate spatial genetic structure surrounding APP. A GAMM is simply a generalized linear mixed model (GLMM) in which part of the linear predictor is specified in terms of smooth (nonlinear) functions of covariates (Lin & Zhang 1999). No adjustment is required to GLMM methods (beyond the inclusion of the smooth term[s]) to fit a GAMM (Wood 2006). GAMMs are extensions of generalized additive models (GAMs; Hastie & Tibshirani 1986) in which ≥ 1 random effect is included in addition to fixed effects (Wood 2006). GAMs and GAMMs are appropriate for analysing spatial genetic patterns because they are flexible, semi- or nonparametric regression models that can be used to model complex, nonlinear relationships between response and predictor variables (Snäll *et al.* 2004). Predictor variables are specified in terms of smooth functions, in our case these were thin-plate regression splines, for which the exact parametric form is unknown (Wood 2006). The smoothing functions (splines) fit curves to nonlinear trends between the response and predictor variables; however, if the relationship with a given predictor variable is better modelled as linear (i.e. estimated degrees of freedom = 1), the term can be included as a parametric fixed effect (Wood 2006). We used GAMMs (rather than GAMs) to allow for the inclusion of pack as a random effect, to account for the social structure of wolves and coyotes

in our models. Mixed models are increasingly being used to analyse ecological data that is hierarchical in nature, such as when individuals are sampled from groups, to avoid violating the assumption of independence among samples required for regression (Bolker *et al.* 2009). Previous studies of wolf ecology have also adopted mixed-modelling regression approaches by specifying pack as a random effect (e.g. Hebblewhite & Merrill 2008).

The GAMMs differed from the sPCA in that we used % eastern wolf ancestry of resident animals as the response variable to explicitly investigate the spatial distribution of these highly assigned and admixed wolves in and around APP. Specifically, we investigated whether the hybrid zone extending out from APP into adjacent areas: (i) showed a cline, a mosaic pattern or elements of both and (ii) whether the pattern was similar in shape and steepness to the west, south and northwest of APP. We predicted that if the hybrid zone adjacent to APP was clinal, the relationship between space and wolf ancestry would be approximately linear and a simple distance variable would explain most of the variation. However, if the pattern was a mosaic, we predicted that the relationship would be modelled better by a spatial variable that allowed for more complex, discontinuous patterns between space and genotype.

We transformed the proportional response variable using the logit transformation ($\ln y/[1 - y]$) to map admixture proportions monotonically to the whole real line ($-\infty, \infty$) and to meet assumptions of regression modelling (Warton & Hui 2011). We conducted these spatial analyses with two model sets. First, we used distance from the centre of APP (hereafter distance) to the centroid of each animal's home range as the continuous independent variable, entered into the model as a smooth (nonparametric) predictor of % eastern wolf ancestry. Next, we substituted the distance variable for a smooth interaction term between easting and northing metric spatial coordinates (hereafter space) to assess whether this variable improved model fit and identified more complex spatial-genotype patterns. Spatial coordinates can be included in regression models as independent variables to detect (and account for) spatial autocorrelation in the response variable (Beale *et al.* 2010). Thus, we used the spatial covariate to model spatial genetic structure of eastern wolf ancestry. We also included a random effect of pack in all models to account for the fact that we sampled (1–4) individuals from different packs across the study area. All GAMMs (and underlying GLMMs) were estimated using restricted maximum likelihood methods that produce less-biased estimates of variance components for random effects in mixed models than traditional maximum likelihoods (Wood 2006; Bolker *et al.* 2009). We

conducted an overall analysis (all study units) to compare models with the distance and space variables and then conducted analyses with data from APP and each of the adjacent study units separately to model the genotypic patterns extending from APP into each adjacent area with distance and space and to consider differences in these patterns. All distance and space models only contained a single predictor term (distance or space), and we assessed fit between pairs of models with the space or distance variables using Akaike's Information Criteria corrected for small samples (AIC_c) and the difference between AIC_c values (ΔAIC_c , Burnham & Anderson 1998). Models with $\Delta AIC_c < 2$ are generally considered to be plausible competing models (Burnham & Anderson 1998). We determined the amount of variation explained by each model using adjusted R^2 values. We included all data from our main population genetics analyses, except that we substituted single breeding males or females for sibling pairs ($n = 4$) and removed single pups from packs that also contained a parent ($n = 3$) or a sibling ($n = 1$). This was done to avoid including any closely related animals from the same pack in our analyses to further ensure independence between samples.

Landscape analysis

Next, we extended the GAMMs to test hypotheses regarding the influence of prey availability [moose (*Alces alces*) and deer (*Odocoileus virginianus*)] and fragmentation/human disturbance (road densities) on the distribution of genotypes in the APP area to investigate the environmental conditions underlying spatial genetic structure. We estimated mean moose density across our study area, and within home ranges of wolves and coyotes, using aerial survey data collected by the Ontario Ministry of Natural Resources (OMNR; see estimation details in Appendix S1, Supporting information). We used a Geographic Information System (GIS) layer of deer wintering areas, compiled and digitized by OMNR and intersected these with wolf and coyote home ranges to calculate the proportion of the home range comprising deer wintering habitat as an index of winter deer availability. We estimated road densities (km/km²) for each wolf and coyote range by developing separate layers for primary, secondary and tertiary roads. Primary roads were paved roads with relatively high traffic volume classified as freeways, expressways or highways. Secondary roads were mostly paved and were classified as arterial, local/street or collector roads, except for a few major gravel logging roads in APP that received relatively high traffic volume and allowed speeds of >50 km/h. Tertiary roads were unpaved roads and trails that received light traffic, mostly from

recreational vehicles and hikers. Additional details of collection, analysis and sources of all data used to develop environmental variables are provided in Appendix S1, Supporting information.

As discussed earlier, harvest was illegal within APP and the surrounding buffer area, and we found no evidence of illegal harvest within the APP study unit despite monitoring survival and cause-specific mortality of >100 radio-instrumented canids in APP between 2006 and 2011 (J. Benson & B. Patterson, unpublished). Given that tertiary roads were smaller, unpaved roads, we assumed their effect on wolves and coyotes would be mostly by providing access to hunters and trappers into otherwise remote areas outside of APP. Therefore, we included an interaction term between tertiary roads and harvest protection status to test the hypothesis that access to harvest (via tertiary roads) would influence wolf-coyote occurrence and ancestry differently in areas with (APP) and without harvest protection (other study units). We included tertiary road density as a nonparametric (smooth) variable and modelled the interaction with a categorical, parametric term for study unit (bivariate term, with study units outside of APP pooled) using the 'by' command in the 'mgcv' package in R. We also included study unit as a parametric main effect in all models retaining the interaction term to account for the fact that smooth terms are subject to a centring constraint, which was not required in this case because of the interaction with a factor variable (Wood 2006). We included pack as a random effect in all landscape models to account for the fact that we sampled 1–4 individuals from different packs.

We used % eastern coyote ancestry (logit transformed) of adult, resident animals as the response variable (inverse was % wolf), under the assumption that eastern and gray wolves would be more similar in their environmental associations than gray wolves and coyotes. This allowed us to primarily compare landscape associations of eastern wolves and coyotes (from which most ancestry of individuals in our sample was derived), without excluding data from individuals with gray wolf ancestry (which represented a smaller proportion of *Canis* ancestry). We conducted two landscape analyses in a hierarchical manner because we were interested in modelling landscape-genotype relationships: (i) across the entire study area including APP and (ii) across all areas outside APP. APP is primarily inhabited by eastern wolves (Rutledge *et al.* 2010) and has higher moose densities, fewer deer wintering areas and lower primary and secondary road densities than surrounding areas (McLoughlin *et al.* 2011). Thus, the analysis restricted to study units adjacent to APP focused on areas characterized by a greater diversity of *Canis* genotypes and more heterogeneous landscape

conditions, such that the results would not be influenced by the more homogenous, protected wolf population and landscape of APP.

In addition to the prey availability and road density variables, we included the spatial covariate (interaction term between easting and northing spatial coordinates, described above) to account for spatial autocorrelation inherent in spatial data sets (Beale *et al.* 2010). We started with the full model (all variables included) and decided which variables to drop following the methods of Wood & Augustin (2002) and Parra *et al.* (2011), modified slightly as we used AIC_c scores rather than generalized cross validation scores. First, we sequentially considered variables as candidates to be dropped based on estimated degrees of freedom (e.d.f.) near the lower limit of 1. Second, we assessed whether zero was included in the confidence interval across the entire range of the predictor variable. Third, we re-ran the model without the variable being considered to determine whether a lower AIC_c score was achieved (indicating improved model fit). If all three criteria were met, we dropped the variable and considered additional variables that were candidates for removal. The landscape analyses were restricted to adult (≥ 2 years old) animals ($n = 85$) in packs ($n = 47$) for which we had sufficient GPS telemetry data to reliably estimate home ranges. We only had sufficient telemetry data to estimate home ranges and associated landscape variables, for two packs in WMU47; thus, the landscape analyses primarily model ancestry-landscape relationships in APP, WMU49 and KH.

Morphological analysis

We applied a correction factor to standardize body mass data because study animals often gained substantial weight during winter in our study area, which has been noted previously for wolves and coyotes (Pouelle *et al.* 1995). The mean increase in weight from nonwinter (April–November) to winter (December–March) captures for individuals captured during both periods was 3.9 kg (SE = 0.86, $n = 11$). Thus, we subtracted and added 2 kg to winter and nonwinter weights, respectively, to standardize weights across seasons for our analyses. We only included weights from adults (≥ 2 years old) in our analyses. For body length, we also included data from yearlings because skeletal growth ceases between 12 and 14 months for wolves (Kreeger 2003). We conducted two separate analysis of variances (ANOVA) to compare mass and length between genotype classes. Genotype classes in our analysis included eastern wolves, coyotes and eastern wolf \times coyote hybrids. The response variables were mass or length, and we tested for effects of sex, genotype class

and sex \times genotype class interactions. If interactions between sex and genotype class were not significant, we conducted post hoc testing between different genotype classes with Tukey's HSD. We considered all tests to be significant if $P < 0.05$ and marginally significant if $0.10 > P > 0.05$. All statistical tests for morphological analyses were conducted using R. We excluded data from highly assigned and admixed gray wolves from ANOVA tests because of small and unbalanced sample sizes. Although our remaining data were also unbalanced between some levels, the 'Tukey HSD' command in R incorporates an adjustment for mildly unbalanced data. The potential consequence of severely unbalanced data with ANOVA is a lack of power (Faraway 2002), which may have made our tests conservative in some cases.

Results

Number of genetic populations: structure and PCA

The Bayesian analysis in Structure provided support for three genetic clusters in the Algonquin Provincial Park (APP) region (Fig. 2, Table S1, Supporting information) and indicated admixture between all 3 (Fig. 3b). We interpreted the three clusters as distinguishing between gray wolves, eastern wolves and coyotes. There was also strong support for two genetic clusters, which we interpreted as the distinction between gray wolves and eastern wolves/coyotes (Figs 2 and 3a, Table S1, Supporting information). The K -means procedure, following principal components analysis (PCA), also showed strong support for $K = 3$ (Fig. 2, Table S1, Supporting information). The PCA results showed two main axes of variation, which explained 6.7% and 4.4% of the variation respectively (Fig. S1, Supporting information). We interpreted these two axes to represent variation between gray wolves and eastern wolves/coyotes (PC1) and eastern wolves and coyotes (PC2; Figs 4 and S1, Supporting information). Remaining axes each explained $\leq 3.6\%$ of remaining variation and were not easily interpreted biologically.

Individual admixture

Individual admixture proportions of resident individuals in the APP area (and the NEON outgroup) at $K = 2$, 3 and 4 are shown in Fig. 3. Given the support for both 2 and 3 genetic clusters in the data, we made individual assignments at $K = 3$. These assignments allowed us to address subsequent hypotheses regarding morphology, spatial genetic structure and landscape associations of gray wolves, eastern wolves and coyotes. PCA corroborated 90% of the original assignments made

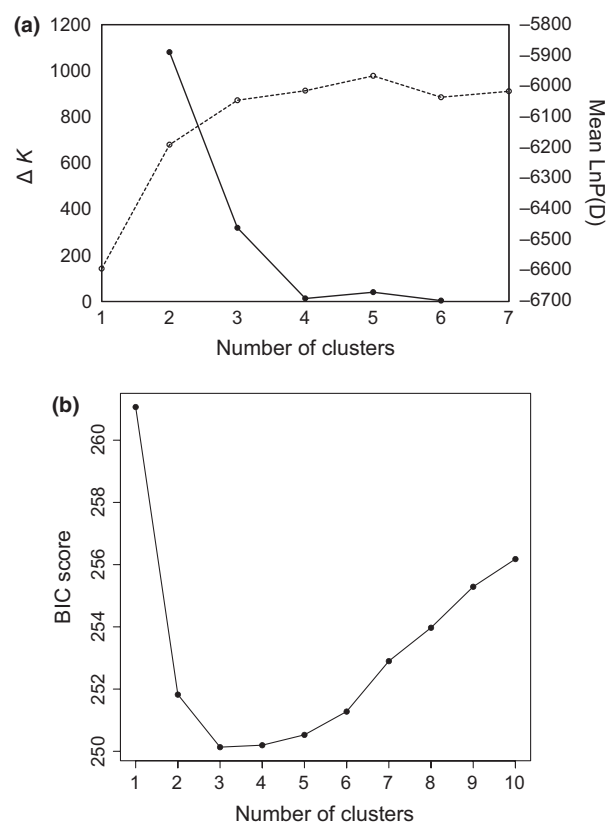


Fig. 2 Results of two genetic Structure analyses to evaluate support for the number of genetic populations (K) in the data. (a) Program Structure analysis showing mean $\ln P[D]$ (dotted line) and ΔK (solid line) for $K = 1-7$, (b) K -means procedure showing Bayesian Information Criteria (BIC) for $K = 1-10$. The strongest supported number of clusters should be the K with the minimum number of clusters after which the BIC value increases or decreases by a negligible amount (Jombart *et al.* 2010).

based on Structure Q -values (at $K = 3$) and our 80% threshold criteria. We also used the PCA results to reclassify 12 animals from their original assignment (Figs 4a and S2, Supporting information). Using this procedure, eight animals were moved from highly assigned to admixed classes, three animals were moved from admixed to highly assigned classes, and one animal was moved from an admixed class between two clusters to the admixture class between three clusters (Figs 4a and S2, Supporting information). PCA indicated that no animals should be moved from one highly assigned class to another. Additionally, four animals had Q scores of < 0.8 for all groups and either > 0.2 for all groups ($n = 2$) or < 0.2 for two groups ($n = 2$), which could have suggested admixture between three groups. PCA indicated these were admixed between two groups ($n = 3$) or were highly assigned to a single group ($n = 1$; Figs 4a and S2, Supporting Information). We

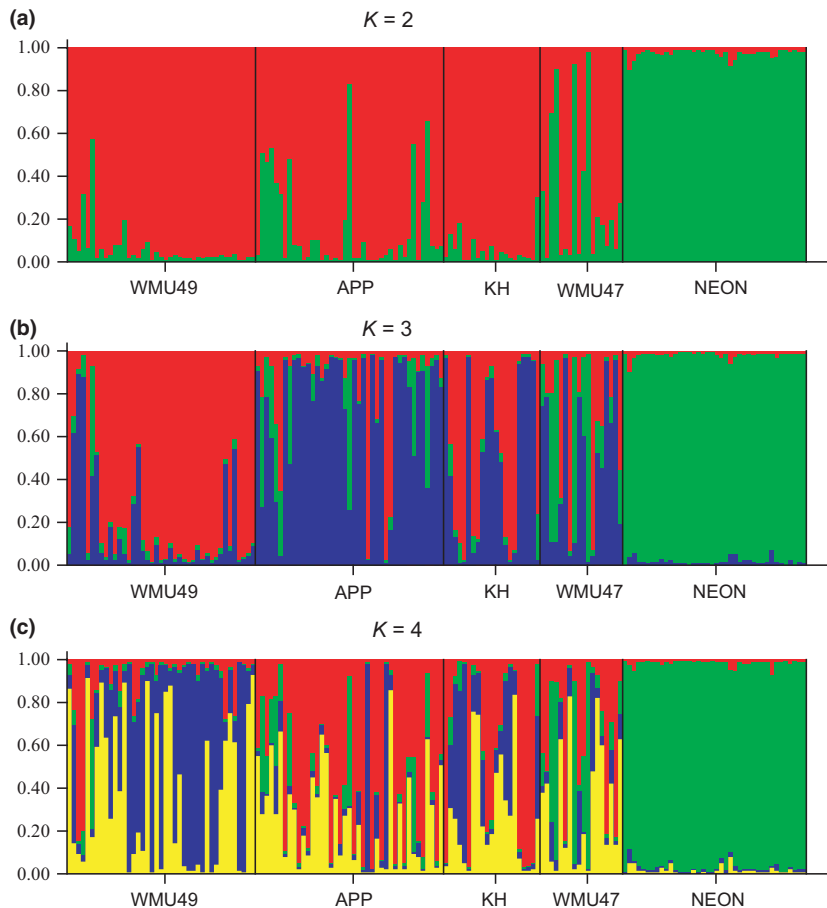


Fig. 3 Bar plots from Structure individual assignments at a range of potential number of genetic clusters ($K = 2-4$). Each bar represents an individual and individuals are grouped by study units (WMU49, APP, KH, WMU47) and the outgroup (NEON). (a) At $K = 2$, we interpret red and green portions of bars as identifying eastern wolf/coyote and gray wolf ancestry respectively. (b) At $K = 3$, we interpret blue, red and green portions of bars as identifying eastern wolf, coyote and gray wolf ancestry, respectively. (c) Our analyses did not strongly support $K = 4$, and we do not speculate on individual assignments with this number of clusters.

also re-ran the PCA after excluding all individuals that were classified as hybrids to provide graphical representation of genetic variation contained in PC1 and PC2 for only the three distinct *Canis* types: gray wolves, eastern wolves and coyotes (Fig. 4b).

Animals in APP were predominantly eastern wolves with smaller numbers of coyotes and hybrid animals, mostly with gray wolf admixture (Table 1, Fig. 5). Conversely, animals in WMU49, ~25 km to the west, were predominantly coyotes and eastern wolf \times coyote hybrids, with fewer gray wolf \times eastern wolf hybrids ($n = 2$), gray wolf \times coyote hybrids ($n = 2$) or eastern wolves ($n = 1$, Table 1, Fig. 5). Animals in KH were a relatively balanced mix of eastern wolves, coyotes and admixed individuals between these two groups (Table 1, Fig. 5). Finally, WMU47 had a mixture of highly assigned and admixed individuals from all three genetic clusters (Table 1, Fig. 5). Genotype frequencies differed across study units ($P < 0.001$, Fisher's exact test; highly assigned and admixed gray wolves pooled because of small samples).

The 90% credible regions calculated in Structure were wide for some admixed individuals, even ranging from 0 to 1 in some cases (Table S2, Supporting information), a

phenomenon noted by previous studies of wolves and coyotes (Wheeldon *et al.* 2010; Bohling & Waits 2011). However, highly assigned animals generally had much narrower credible regions and many ranged from >0.8 to 1.0 for their group of assignment (Table S2, Supporting information). Previous testing of Bayesian credible regions from Structure with individuals of known ancestry suggested they may be overly conservative in terms of overstating uncertainty of q -value estimates (Bohling & Waits 2011); nevertheless, the wide credible regions from Structure for some individuals highlighted the importance of corroborating individual assignments with additional analysis. Q scores (mean and standard deviations from 10 runs at $K = 3$), 90% credible regions and original and final assignments for all individuals in the main analysis are provided in Table S2 (Supporting information). We also provide assignments for all individuals at $K = 2$ based on the run with the highest $\text{Ln}(P)D$ and lowest variance (Table S3, Supporting information).

Spatial genetic structure: sPCA and spatial modelling

Using sPCA, we found significant global ($P = 0.002$) but not local ($P = 0.780$) structure across our study area.

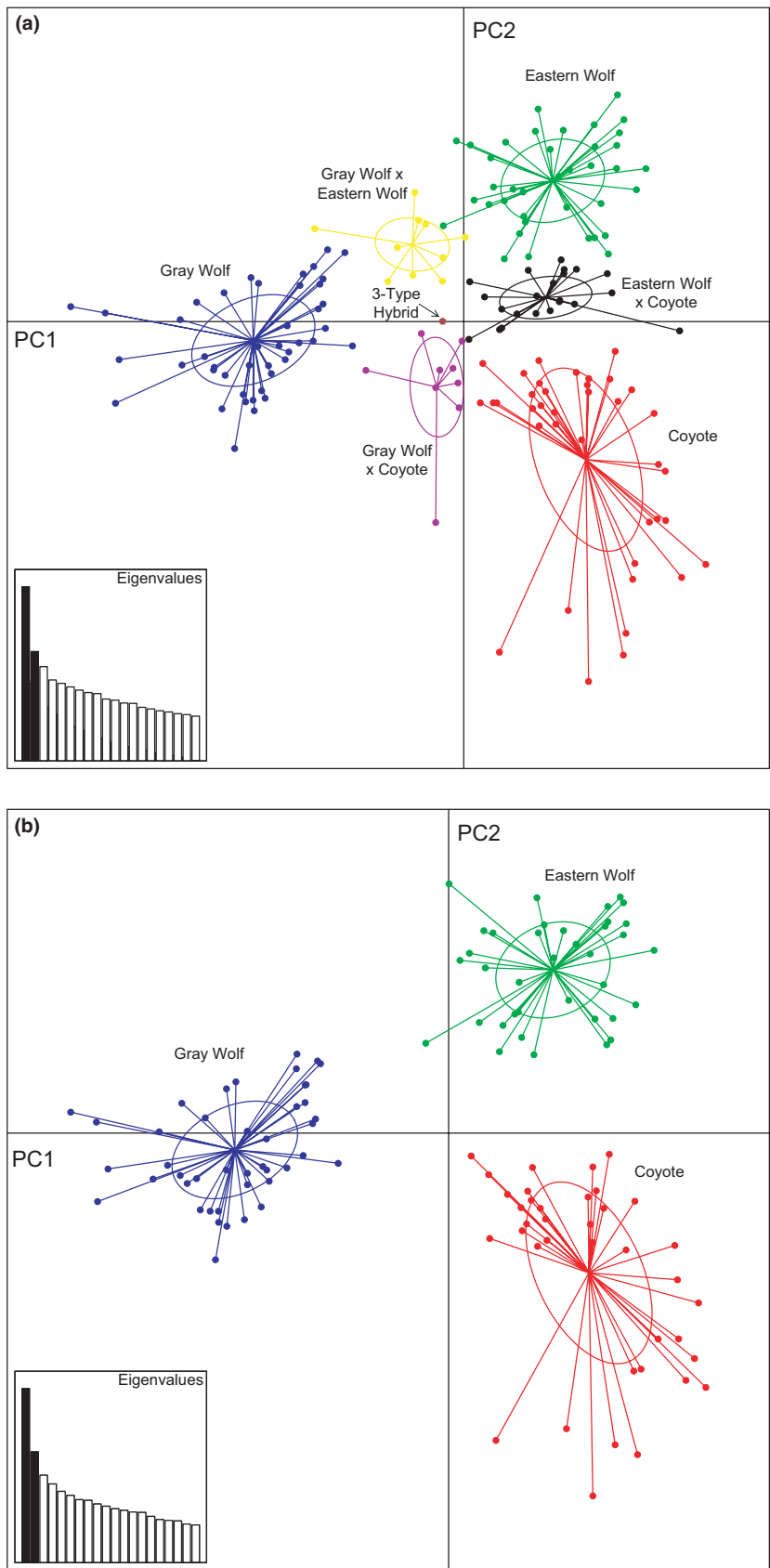


Fig. 4 Individual wolves, coyotes and hybrids in and adjacent to APP ($n = 121$) and the NEON outgroup ($n = 40$) arranged along axes 1 and 2 of principal components analysis (PCA), which explained 6.7% and 4.4% of the total variation, respectively. PCA was used to corroborate individual assignments to genetic clusters and admixed categories made at $K = 3$ with results from program Structure. Different genotype classes are represented with different colours, and 95% confidence ellipses are shown for each class. The two plots show: (a) all individuals in main analysis and (b) all individuals highly assigned to distinct clusters ($n = 119$) to provide graphical representation of the distinct gray wolf, eastern wolf, coyote clusters at $K = 3$.

Table 1 Proportions and numbers of resident and breeding animals of each genotype in Algonquin Provincial Park (APP), Wildlife Management Unit 49 (WMU49), Kawartha Highlands (KH) and WMU47 in Ontario, 2004–2010

	APP				WMU49				KH				WMU47			
	Residents		Breeder*s		Residents		Breeder*s		Residents		Breeder*s		Residents		Breeder*s	
	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
Eastern Wolf	62.5	25	62.5	20	2.4	1	3.3	1	38.1	8	44.4	4	16.7	3	50.0	3
Coyote	5.0	2	6.3	2	64.3	27	60.0	18	33.3	7	22.2	2	11.1	2	0	0
Gray Wolf	0	0	0	0	0	0	0	0	0	0	0	0	16.7	3	16.7	1
Coyote × Eastern Wolf	7.5	3	3.1	1	23.8	10	26.7	8	19.0	4	22.2	2	27.8	5	33.3	2
Gray × Eastern Wolf	17.5	7	18.8	6	4.8	2	6.7	2	0	0	0	0	11.1	2	0	0
Gray wolf × Coyote	7.5	3	9.4	3	4.8	2	3.3	1	9.5	2	11.1	1	11.1	2	0	0
3-way hybrid	0	0	0	0	0	0	0	0	0	0	0	0	5.6	1	0	0

*Not all breeding animals were identified; % is proportion of all known breeders.

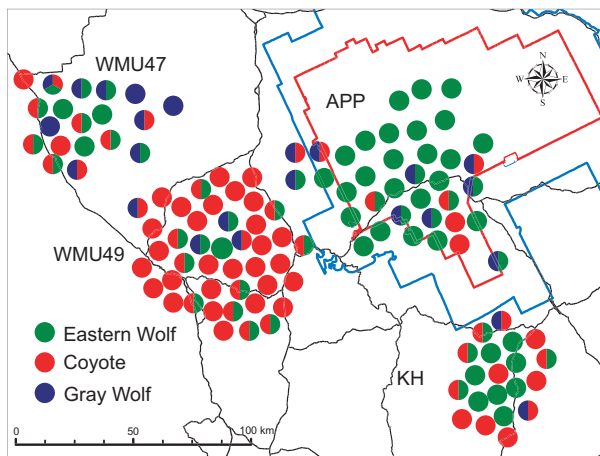


Fig. 5 Study area with resident individuals plotted (approximately) at home range centroids with pie charts showing genotypes based on individual assignment to genetic clusters using Structure and PCA. >1 colour in pie charts indicates admixture. Pie charts are simplified to show 100% ancestry for highly assigned individuals, and 50–50% or 33–33–33% for individuals admixed between 2 or 3 parental clusters, respectively. Also shown are major roads (black lines), APP boundary (red line) and harvest ban buffer area boundary (blue line).

The global structure revealed the spatial genetic patterns extending from APP to the west, south and northwest (Fig. 6). Genetic differentiation to the west between APP and WMU49 was great and the cline was very steep; however, the differentiation was much more gradual to the south (into KH) and northwest (into WMU47; Fig. 6).

Eastern wolf ancestry was not well modelled by distance from the centre of APP (distance) across the study area ($R^2 = 0.12$, $AIC_c = 516.9$, $n = 113$; Fig. 7b). The interactive term with easting and northing spatial coordinates (space) improved model fit ($R^2 = 0.32$, $AIC_c = 507.4$, $n = 113$) and indicated there was significant

spatial genetic structure in eastern wolf ancestry throughout the study area (Fig. 7a). When considering study units separately, the model with data from WMU49 and APP suggested a steep cline of decreasing eastern wolf ancestry extending west from APP with distance as a predictor of ancestry ($R^2 = 0.34$, $AIC_c = 328.6$, $n = 75$). However, spatial structure from APP to WMU49 was better modelled with space ($R^2 = 0.48$, $AIC_c = 321.7$, $n = 75$) than it had been with distance. Model fit with distance was poor for KH ($R^2 = 0.05$, $AIC_c = 277.5$, $n = 59$) and improved with space ($R^2 = 0.29$, $AIC_c = 273.6$, $n = 59$), suggesting that the spatial structure was not well modelled as a cline into KH. WMU47 was not well modelled with distance ($R^2 = 0.09$, $AIC_c = 249.9$, $n = 57$) or space ($R^2 = 0.19$, $AIC_c = 251.6$, $n = 57$). Although the model with space explained more variation in eastern wolf ancestry in WMU47 than the model with distance, ΔAIC_c was <2 suggesting that neither model was substantially better.

Landscape analyses

The best landscape model explaining variation in coyote ancestry in resident, adult animals across the entire study area (including APP, $R^2 = 0.57$, $n = 85$) retained additive, linear effects of moose density ($F = 6.9$, e.d.f. = 1, $P = 0.010$, Fig. 8a) and the spatial covariate ($F = 1.5$, e.d.f. = 2, $P = 0.216$), as well as the positive, nonlinear effect of secondary road density ($F = 5.1$, e.d.f. = 1.7, $P = 0.013$; Fig. S3b, Supporting information). There was also a significant interaction between tertiary road density and harvest protection, as there was a positive, linear effect of tertiary road density outside of APP ($F = 5.0$, e.d.f. = 1, $P = 0.029$), but inside of APP there was not a significant relationship between tertiary road density and coyote ancestry ($F = 1.2$, e.d.f. = 2.4, $P = 0.312$; Fig. S4, Supporting Information). When data

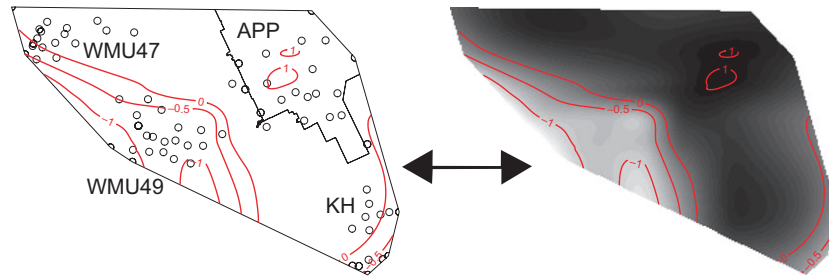


Fig. 6 Maps of spatial genetic Structure from sPCA analysis for 121 resident wolves and coyotes in APP region. Both maps are of same area and represent results of same analysis, shown at different scales of genetic differentiation. Map on left shows sample locations (circles), APP boundary and red contour lines of major genetic differences. Map on right shows contours of finer genetic differences in dark to light shading.

from APP were excluded, the best model explaining variation in the degree of coyote ancestry in resident, adult animals ($R^2 = 0.40$, $n = 51$) retained the positive, nonlinear effect of secondary road density ($F = 4.2$, e.d.f. = 1.9, $P = 0.024$, Fig. 8b) on coyote ancestry. The negative linear effect of moose density ($F = 4.4$, e.d.f. = 1, $P = 0.041$, Fig. S3a, Supporting information), the positive linear effect of tertiary road density ($F = 6.2$, e.d.f. = 1, $P = 0.016$, Fig. 8c) and the spatial covariate ($F = 1.9$, e.d.f. = 2, $P = 0.168$) were also retained.

Morphological analyses

Body mass and length were different between males and females (Weight: $F_{65,1} = 25.8$, $P < 0.001$; Length: $F_{92,1} = 7.8$, $P = 0.007$) and between genotype classes (Weight: $F_{65,2} = 32.3$, $P < 0.001$; Length: $F_{92,2} = 26.2$, $P < 0.001$), but there was not a significant interaction between sex and genotype class for mass ($F_{63,2} = 0.3$, $P = 0.713$) or length ($F_{90,2} = 0.1$, $P = 0.873$). Eastern wolves were heavier than both coyotes ($P < 0.001$) and coyote \times eastern wolf hybrids ($P < 0.001$, Table 2). However, there were not significant differences in mass between coyotes and coyote \times eastern wolf hybrids ($P = 0.121$). Eastern wolves were significantly longer than both coyotes ($P < 0.001$) and coyote \times eastern wolf hybrids ($P = 0.001$), and coyote \times eastern wolf hybrids were significantly longer than coyotes ($P = 0.032$, Table 2). When considering all genotype classes (including gray wolves and admixed gray wolves), mean body mass and length for each genetic cluster followed a decreasing gradient from gray wolf to eastern wolf to coyote, with associated hybrids generally exhibiting intermediate mean morphological characters, providing additional (nonstatistical) support for our hypothesis (Table 2).

Discussion

We have demonstrated that three *Canis* types inhabit the region in and adjacent to Algonquin Provincial Park

(APP): Algonquin-type eastern wolves, eastern coyotes and admixed gray wolves. Our genetic analyses showed support for 2 and 3 genetic clusters in the sample of resident individual wolves and coyotes, which were distributed in home ranges spanning a mostly contiguous area across western APP and adjacent areas to the west, northwest and south. We interpret support for two genetic clusters in our Structure analysis as identifying broad structure between gray wolves and eastern wolves/coyotes. However, our analyses also clearly supported an additional level of population structure at $K = 3$, which we interpret as identifying the additional distinction between eastern wolves and coyotes. The clusters of the three highly assigned *Canis* types are easily visualized by examining axes of genetic variation identified with principal components analysis (PCA) (Fig. 4). The PCA also identified a relatively high proportion (36%) of admixed individuals that were arranged along the axes at intermediate positions between the distinct clusters (Fig. 4a). The presence of multiple, valid layers of genetic structure in a given data set is not unusual, and indeed, many previous studies of *Canis* genetic structure have found support for multiple K -values in single data sets (e.g. Koblmüller *et al.* 2009; Fain *et al.* 2010; Rutledge *et al.* 2010; von Holdt *et al.* 2011). We based our subsequent analyses on assignments made at $K = 3$ because, after showing support for this number of clusters in the molecular data, this level of genetic structure allowed us to test morphological and ecological hypotheses regarding eastern wolves, coyotes and gray wolves in the APP hybrid zone. At $K = 2$ all highly assigned eastern wolves and coyotes were assigned to a single class (Table S3, Supporting information). Thus, the morphological and ecological differences we documented between eastern wolves and coyotes clearly support the contention that an additional, biologically meaningful level of *Canis* population structure exists in the APP region.

Elucidating genetic structure in hybrid zones between closely related species and populations is a difficult and

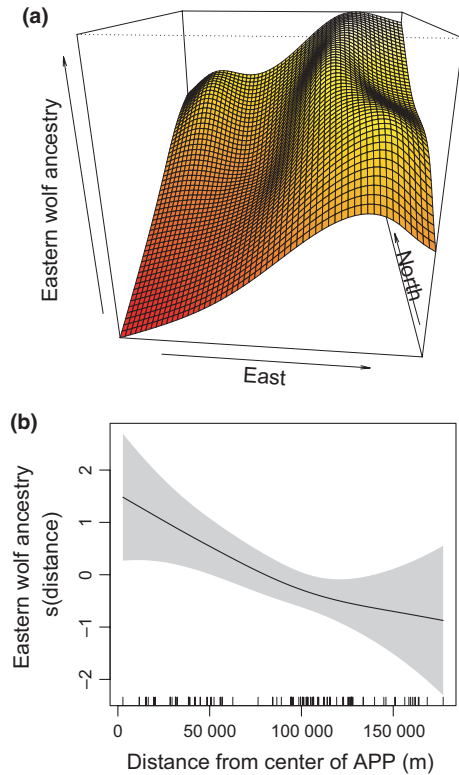


Fig. 7 Results of two competing spatial genetic models of eastern wolf ancestry in the APP region based on generalized additive mixed models (GAMMs). (a) 3-D perspective plot of spatial GAMM ($R^2 = 0.32$, e.d.f. = 7.9, $P = 0.001$, $n = 113$) showing % eastern wolf ancestry (z-axis) as function of a smooth interactive term with easting (east; x-axis) and northing (north; y-axis) spatial coordinates. Yellow colour and higher peaks represent greater levels of eastern wolf ancestry, orange is intermediate, and red is lower levels of eastern wolf ancestry. WMU47, APP, KH, WMU49 study units are in NW, NE, SE, SW portions of the plot, respectively. (b) Distance GAMM ($R^2 = 0.12$, e.d.f. = 1.5, $P = 0.015$, $n = 113$), showing % eastern wolf ancestry as a smooth function (s) of distance from APP centre (in metres, x-axis) with data from all study units. On y-axis, eastern wolf ancestry is centred on 0, with positive values indicate increasing eastern wolf ancestry, negative values indicate decreasing eastern wolf ancestry. Shaded area is 95% confidence interval around predicted trend in ancestry, and vertical bars on x-axis indicate sample locations.

uncertain endeavour. Our use of 12 microsatellite loci provided lower resolution than would have been possible with larger numbers of loci, or perhaps different markers, in terms of correctly identifying the ancestry of individuals with genotypes resulting from complex hybridization patterns. Greater numbers of microsatellite loci (≥ 48) are sometimes necessary to distinguish between parental types and advanced backcrossed individuals in hybrid zones (Vähä & Primmer 2006). Additionally, the use of single nucleotide polymorphisms (SNPs) is increasing and may provide researchers with

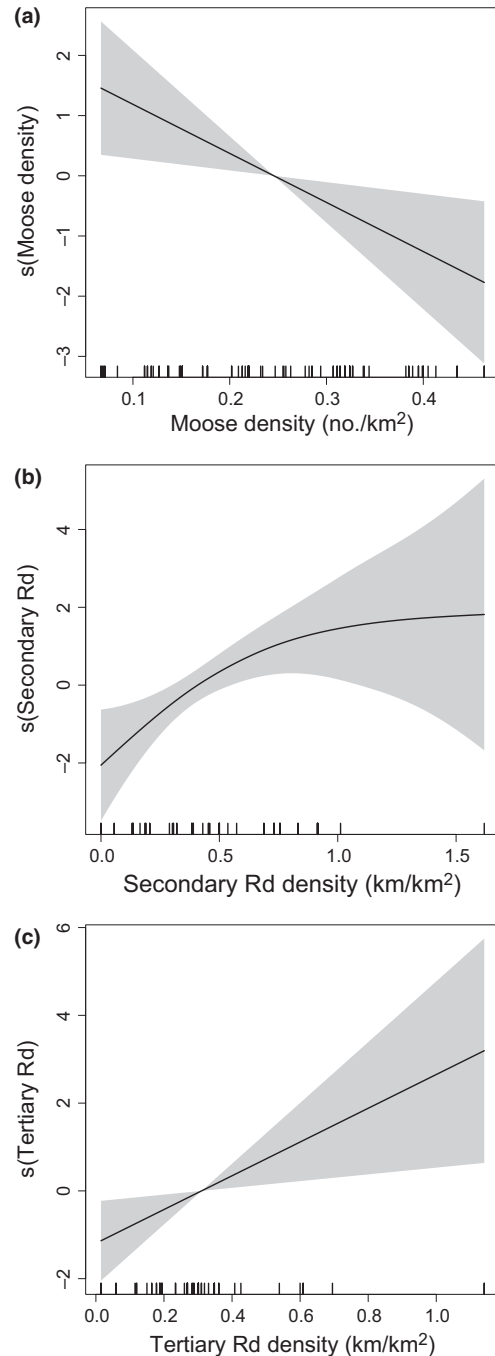


Fig. 8 Relationships between % coyote ancestry and: (a) mean moose density ($P = 0.01$, $n = 84$), (b) secondary road density ($P = 0.02$, $n = 51$) and (c) tertiary road density ($P = 0.02$, $n = 51$) throughout the home ranges of resident adult wolves and coyotes across the study area (a) or study units adjacent to APP (b and c) as predicted by generalized additive mixed models. Y-axis shows % coyote ancestry as a smooth function (s) of the independent (environmental) variables centred on 0 where positive values indicate increasing coyote ancestry, negative values indicate decreasing coyote ancestry. Shaded area is 95% confidence interval around predicted trend, and vertical bars on x-axis indicate sample locations.

Table 2 Mean body mass and length of wolves and coyotes from in and adjacent to Algonquin Provincial Park in Ontario, Canada, 2004–2010. Also shown are standard errors (SE) and sample size (*n*)

	Mass (kg)*						Length (cm)†					
	Females			Males			Females			Males		
	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>
Coyote	17.8	0.6	11	22.0	1.1	10	97.3	1.6	17	102.2	1.9	14
Eastern Wolf × Coyote	19.6	0.8	7	24.3	1.0	7	103.0	2.4	10	105.8	1.6	11
Eastern Wolf	25.0	0.8	19	28.2	1.0	15	109.3	1.3	24	113.0	1.8	21
Gray × Eastern Wolf	24.7	2.0	3	34.4	1.0	5	111.0	2.2	6	120.7	1.7	5
Gray Wolf	ND	–	–	36.8	3.2	3	ND	–	–	119.0	0	1
Gray Wolf × Coyote	ND	–	–	28.2	2.7	5	98.0	0	1	113.3	3.8	5

ND, no data.

*We subtracted and added 2 kg to winter and nonwinter weights, respectively, to account for increases in weight during winter (mean increase = 3.9 kg).

†Length from tip of nose to base of tail.

much greater resolution for untangling complex population structure, as analysis of thousands of loci, or even whole-genome analyses are becoming possible (Morin *et al.* 2004; Helyar *et al.* 2011; von Holdt *et al.* 2011). Although using additional loci or markers may have allowed us to more accurately identify genetic ancestry of individuals, our study also employed many powerful field techniques (e.g. GPS telemetry, aerial tracking) that are rarely used in combination with detailed molecular analysis. We limited our genetic inferences to resident, breeding packs and showed that genetic distinctions, observed and corroborated with multiple analyses, manifested in both differences in morphology and associations with different landscape attributes. Thus, although we used a relatively modest number of microsatellite loci, our overall approach, using genetic, morphologic, demographic and behavioural data, represented a more balanced and powerful approach to molecular ecology than studies employing large numbers of loci, but with no means of assessing whether the inferences were biologically meaningful. For studies seeking to provide practical information for the conservation and management of wild populations, verifying that genetic inferences are biologically significant is an important, if often overlooked, consideration.

Despite extensive admixture, highly assigned individuals of the three *Canis* types exhibited genetic and morphologic differences within the relatively limited geographic area in and adjacent to APP. The portion of the hybrid zone within our study area contained mostly eastern wolves, coyotes and admixed individuals with varying levels of eastern wolf, coyote and gray wolf ancestry. However, there were also resident, breeding gray wolves in the northernmost study unit (WMU47), suggesting that gray wolves probably disperse into the

APP region via north-eastern Ontario. Resident, admixed gray wolves were present in the other three study units, but highly assigned gray wolves were not found. The southernmost study unit (KH) was primarily inhabited by eastern wolves, coyotes and coyote × eastern wolf hybrids, suggesting that south of APP the hybrid zone mostly comprises animals whose ancestry is derived from only two genetic clusters.

Our analyses provide novel information regarding the spatial structure of areas adjacent to APP and have important implications for conservation of eastern wolves in unprotected landscapes. The steep cline we identified from APP into WMU49, over which the dominant genotype changed abruptly from eastern wolf to coyote and hybrid, shows the dramatic influence this large protected area exerts on fine-scale *Canis* genetic structure. The genotypic patterns to the south and northwest appear clinal in the transition zone between APP and the surrounding matrix, a perception that was probably accentuated by considering hybrid zone structure along an abrupt change in environmental conditions (Bridle *et al.* 2002; Ross & Harrison 2002). However, there were patches in KH and WMU47 that were more similar genetically to APP than to WMU49 indicating that the hybrid zone may be better characterized as a mosaic in these areas. Thus, our results show the importance of APP as the population core for eastern wolves, but also indicate that eastern wolves can inhabit unprotected landscapes where suitable environmental conditions exist.

The three genetically distinct *Canis* types we identified also differed in morphology, as we found a decreasing gradient in body mass and length from gray wolves to eastern wolves to coyotes. We also documented intermediate morphology for hybrids, which is

common in animal hybrid systems (e.g. Grant & Grant 1994; Mavárez *et al.* 2006; but see Ackermann *et al.* 2006), although differences in mean body mass were not statistically significant between coyotes and eastern wolf \times coyote hybrids. Regardless, our results indicate that highly assigned eastern wolves, gray wolves and coyotes retain morphological differences despite extensive hybridization in central Ontario. Additionally, our morphological results are important because they correspond closely with our individual genetic assignments and were consistent with assumptions regarding ancestry of the distinct *Canis* types.

Previous studies have provided detailed analysis of the protected and relatively homogenous eastern wolf population within the boundaries of APP and also compared it with populations across Ontario and Quebec (Grewal *et al.* 2004; Rutledge *et al.* 2010). Grewal *et al.* (2004) compared APP with a study unit to the west referred to as the Magnetawan region, which appeared to overlap with areas in WMU49 and WMU47 (based on their Fig. 2), although little specific information was provided regarding the sample locations. Regardless, Grewal *et al.* (2004) concluded that genetic differentiation between canids in APP and Magnetawan was lower than between APP and the Frontenac Axis (FRAX) southeast of APP. In contrast, we found a steep cline between APP and WMU49 to the west, which was characterized mostly by coyotes and hybrids. Rutledge *et al.* (2010) analysed a subset of the same FRAX samples from Grewal *et al.* (2004), identified them as primarily eastern coyotes and confirmed the earlier results with respect to genetic differentiation between APP and FRAX. We note that the westernmost sample collected in FRAX by the previous studies was collected ~25 km from the home range centroid of the easternmost individual in our sample from KH, and yet our findings were quite different. We found KH to be inhabited by a relatively balanced combination of eastern wolves, coyotes and hybrids, whereas the earlier studies found canids in FRAX to be genetically distinct from APP and to be mostly eastern coyotes and hybrids (Grewal *et al.* 2004; Rutledge *et al.* 2010). Differences in our results may be explained simply by the different sample locations, as despite the proximity of KH and FRAX, there was no overlap between these study units. These different results may also provide additional evidence of the mosaic distribution of the *Canis* hybrid zone in relation to environmental heterogeneity in areas adjacent to APP. However, the samples analysed by these earlier studies were collected during 1995–1998 (see details in Sears *et al.* 2003) which was ≥ 10 years before we sampled DNA from individuals in KH in 2009–2010. This leaves open the possibility that eastern wolf presence has increased in adjacent areas south of APP since the

collection of the samples analysed by Grewal *et al.* (2004) and Rutledge *et al.* (2010), perhaps as a result of increased harvest protection in the buffer area around APP since December 2001. Collection and analysis of contemporary samples from the FRAX region could evaluate this speculation.

The patchwork of eastern wolf genotypes and ancestry we documented outside of APP was influenced by heterogeneous environmental conditions, as animals with higher proportions of wolf ancestry were associated with lower levels of human disturbance (i.e. roads) and higher densities of large ungulate prey (i.e. moose). Across our study area, the environmental conditions and management regulations of APP appear to represent the most suitable current habitat conditions available for eastern wolves. APP was characterized by a lesser degree of anthropogenic habitat fragmentation and road densities than other study units, supported the highest moose densities across the study area (Table S4, Supporting information) and prohibited harvest of wolves and coyotes in the park and surrounding buffer area. APP may be difficult for coyotes to colonize given that smaller prey, such as deer, are relatively scarce in summer and largely absent in winter in western APP (Cook *et al.* 1999). Also, alternative foods such as garbage or the remains of hunted animals are presumably rare relative to adjacent areas. In contrast to the findings of the previous studies, our results suggest that gray wolf admixture may currently be more prevalent in APP than admixture with coyotes. This apparent discrepancy may be due to the fact that we mostly sampled western APP, whereas the previous studies sampled extensively in eastern APP (Wilson *et al.* 2000; Grewal *et al.* 2004; Rutledge *et al.* 2010). Gray wolf admixture could be more prevalent in western APP because of differences in prey base favouring gray wolves, as moose densities are higher in that portion of the park. Western APP is also in closer proximity to north-eastern Ontario, a likely source of gray wolf immigration into the APP region. Alternatively, gray wolf admixture may have increased within APP because these earlier studies and continued genetic sampling of the population should investigate this possibility.

Moose density was likely a better predictor of *Canis* ancestry than deer availability because both wolves and eastern coyotes prey extensively on deer (e.g. Messier *et al.* 1986; Patterson & Messier 2003), whereas wolves are more effective predators of moose (Mech & Peterson 2003; Loveless 2010). Alternatively, as we relied on an index of deer availability, rather than directly estimating density of deer across the study area, greater uncertainty in this variable could have affected our ability to detect significant relationships. The negative relationship between coyote ancestry and moose density

predicted by the models, although significant, was probably weakened by the fact that a few animals outside of APP with relatively high proportions of coyote ancestry occupied areas of high moose density. These areas may have contained alternate, smaller prey, but coyotes may also benefit in such areas by feeding on moose as carrion (Boisjoly *et al.* 2010) and perhaps also by occasional opportunistic predation. In unprotected landscapes, patches with suitable prey availability for wolves may be occupied by coyotes, at least temporarily, as human-caused mortality of wolves may create vacant areas that can be occupied by coyotes, particularly in areas like WMU49 where wolves are rare. In contrast to western wolf-coyote systems where spatial overlap of resident sympatric wolves and coyotes is high (Berger & Gese 2007), resident wolves, coyotes and hybrids in our study area exhibit a high degree of spatial segregation, likely due to more subtle differences in body size and resource use (J. Benson & B. Patterson, unpublished data). Thus, areas of suitable wolf habitat occupied by coyotes may be difficult to colonize by dispersing wolves attempting to settle outside of APP.

Previous studies have found wolf densities to be negatively associated with road densities, particularly in areas where wolf harvest is allowed (Mech *et al.* 1988; Mladenoff *et al.* 1995), whereas coyotes are often abundant in areas with high road densities and associated human disturbance (Gehring & Swihart 2003; Riley *et al.* 2003). However, our findings are unique in indicating that hybridization dynamics between these species are influenced by the density of secondary and tertiary roads. The interaction between tertiary road density and harvest protection, in terms of its effect on *Canis* ancestry, can be understood by considering how secondary and tertiary roads affect wolves and coyotes. Secondary roads probably affect wolves and coyotes both by fragmenting the habitat and also by providing access for hunters and trappers (Thiel 1985; Fuller *et al.* 2003). Tertiary roads probably affect wolves and coyotes primarily by providing access for harvest, as these unpaved roads and trails are probably not a significant source of fragmentation or mortality from vehicle collisions. Where wolves are protected, wolf presence may actually be positively associated with tertiary roads because wolves use linear features such as roads to facilitate rapid movement across rugged terrain (James & Stuart-Smith 2000; Whittington *et al.* 2005). Tertiary road density in our study area was significantly associated with greater coyote ancestry only in areas where harvest was legal, suggesting that eastern wolves may be more susceptible and/or less demographically resilient to trapping and shooting mortality than coyotes. This would be consistent with previously observed relationships regarding the sensitivity of wolves and

resilience coyote populations to human persecution (e.g. Sterling *et al.* 1983; Fritts *et al.* 2003). Indeed, the past introgression from coyotes into the APP wolf population has been linked to high harvest pressure during wolf culls in APP during the 1960s (Rutledge *et al.* 2011). Our results suggest that lower levels of harvest, such as those occurring presently in areas adjacent to APP, also may influence hybridization dynamics between wolves and coyotes. Future studies should compare genotype-specific survival and cause-specific mortality of radio-collared wolves and coyotes to directly test the hypothesis that wolves are more susceptible to harvest than coyotes in unprotected areas adjacent to APP.

Conclusions

Although hybridization between eastern wolves, coyotes and gray wolves has been extensive in the Algonquin Provincial Park (APP) region, it is notable that many (64%) individuals were highly assigned to distinct *Canis* types. Thus, the hybrid zone is not truly bimodal (i.e. mostly genotypes resembling parental types) or unimodal (i.e. mostly hybrids), but is better described as an intermediate or 'flat' hybrid zone with a more balanced mix of highly assigned and admixed individuals (Harrison & Bogdanowicz 1997; Jiggins & Mallet 2000). Bimodal hybrid zones suggest that the species involved possess well-developed, but incomplete, prereproductive isolation mechanisms, whereas unimodal hybrid zones indicate these mechanisms are weak and/or that selection against hybrids is absent (Jiggins & Mallet 2000; Rubidge & Taylor 2004). It follows that an intermediate hybrid zone, such as the one we studied, would have characteristics of both. Indeed, prereproductive barriers probably explain the dominance of eastern wolf genotypes within APP, perhaps with assortative mating as the mechanism (Rutledge *et al.* 2010). However, it is unknown whether eastern wolves in APP generally breed with eastern wolves because of an innate preference, or because the environmental conditions favourable to eastern wolves in APP result in a relatively homogenous population where mating opportunities with other genotypes are limited. Investigating the mating patterns of wolves and coyotes outside of APP would be informative, as eastern wolves would encounter fewer conspecifics and a more diverse range of prospective mates. Also, increased human-caused mortality of wolves and coyotes in harvested areas outside of APP may result in higher rates of mate turnover. Understanding whether the prereproductive mechanisms that have maintained the distinct population in APP are intrinsic or environmentally mediated, and whether they are also exhibited by the patchily

distributed eastern wolves in unprotected landscapes would provide insight into whether these wolves represent viable extensions of the APP population. If these mechanisms are absent at lower densities, the occurrence of highly assigned eastern wolves outside of APP may be ephemeral and largely maintained by regular dispersal from the park.

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J.F. Benson designed research, conducted research, analysed data, wrote manuscript and acquired funding. B.R. Patterson designed research, conducted research, acquired funding and provided guidance during fieldwork, analysis and writing. T.J. Wheeldon conducted laboratory work and provided guidance during analysis and writing.

Data accessibility

mtDNA sequences-GenBank accession nos AY267718, AY267726, AY267731, AY267731, AY267736, FJ687608. Microsatellite, morphological, prey availability and road density data available at DRYAD entry doi:10.5061/dryad.568kn

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Individual wolves, coyotes, and hybrids in and adjacent to APP ($n = 121$) and the NEON outgroup ($n = 40$) arranged along axes 1 and 2 of principal components analysis (PCA), which explained 6.7% and 4.6% of the total variation, respectively.

Fig. S2 Original individual genetic assignments before PCA corroboration procedure with individuals ($n = 161$) grouped by genetic populations and admixed categories.

Fig. S3 Coyote ancestry as a smooth function (s) of: (a) mean moose density ($P = 0.041$, $n = 51$) in study units outside of APP, and (b) secondary road density ($P = 0.013$, $n = 85$) across the study area (including APP) as predicted by generalized additive mixed models.

Fig. S4 Coyote ancestry as a smooth function(s) of tertiary road density showing the significant interaction between tertiary road density and harvest protection as predicted by generalized additive mixed model.

Table S1 Summary data from Structure and K -means procedures for evaluating support for the number of *Canis* genetic clusters in central Ontario in and adjacent to Algonquin Provincial Park, 2004–2011.

Table S2 Q-scores from program Structure at $K = 3$ for each individual ($n = 161$) in main analysis. ID for each individual indicates study unit of residency [AP, Algonquin Provincial Park; KH, Kawartha Highlands; 47, WMU47; 49, WMU49; NE, Northeast Ontario (outgroup)].

Table S3 Q-scores from program Structure inferred at $K = 2$ for each individual ($n = 161$) in main analysis.

Table S4 Mean prey availability and road densities within home ranges (95% fixed kernels) of study animals across the four study units of our study area in and adjacent to Algonquin Provincial Park (2004–2011).

Table S5 Correlation matrix of predictor variables included in generalized additive models (GAMMs) used to investigate associations between *Canis* ancestry and landscape variables in central Ontario, 2004–2011.

Table S6 Mitochondrial DNA haplotypes from individuals included in our main analysis of resident wolves, coyotes, and hybrids from central Ontario inhabiting four study units [Algonquin Provincial Park (APP), WMU49, Kawartha Highlands (KH), and WMU47], 2004–2011.

Table S7 Y-Chromosome haplotypes based on four microsatellites loci (see Appendix S1, Supporting information) from individuals included in our main analysis of resident wolves, coyotes, and hybrids from central Ontario inhabiting four study units [Algonquin Provincial Park (APP), WMU49, Kawartha Highlands (KH), and WMU47], 2004–2011.

Appendix S1 Methods.