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ORIGINAL ARTICLE

Genetic differentiation of eastern wolves in Algonquin Park despite bridging gene flow between coyotes and grey wolves

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Distinguishing genetically differentiated populations within hybrid zones and determining the mechanisms by which introgression occurs are crucial for setting effective conservation policy. Extensive hybridization among grey wolves (*Canis lupus*), eastern wolves (*C. lycaon*) and coyotes (*C. latrans*) in eastern North America has blurred species distinctions, creating a *Canis* hybrid swarm. Using complementary genetic markers, we tested the hypotheses that eastern wolves have acted as a conduit of sex-biased gene flow between grey wolves and coyotes, and that eastern wolves in Algonquin Provincial Park (APP) have differentiated following a history of introgression. Mitochondrial, Y chromosome and autosomal microsatellite genetic data provided genotypes for 217 canids from three geographic regions in Ontario, Canada: northeastern Ontario, APP and southern Ontario. Coyote mitochondrial DNA (mtDNA) haplotypes were common across regions but coyote-specific Y chromosome haplotypes were absent; grey wolf mtDNA was absent from southern regions, whereas grey wolf Y chromosome haplotypes were present in all three regions. Genetic structuring analyses revealed three distinct clusters within a genetic cline, suggesting some gene flow among species. In APP, however, 78.4% of all breeders and 11 of 15 known breeding pairs had assignment probability of $Q \geqslant 0.8$ to the Algonquin cluster, and the proportion of eastern wolf Y chromosome haplotypes in APP breeding males was higher than expected from random mating within the park ($P < 0.02$). The data indicate that Algonquin wolves remain genetically distinct despite providing a sex-biased genetic bridge between coyotes and grey wolves. We speculate that ongoing hybridization within the park is limited by pre-mating reproductive barriers.
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Keywords: assortative mating; conservation; eastern wolf; gene flow; genetic cline; introgressive hybridization

Introduction

Hybridization between animal species is considered by some to be one of the greatest threats to biodiversity (Rhymer and Simberloff, 1996). As more hybrid animal populations are identified, however, hybridization is increasingly recognized as a natural evolutionary process represented as an ongoing ebb and flow of hybridization-speciation events in response to environmental variables over time (Seehausen, 2004; Mallet, 2005). This new outlook challenges how conservation biologists view hybridization and its role in speciation, but remains problematic for setting conservation guidelines (Allendorf *et al*., 2001) because it disrupts the foundation of conservation policies based largely on the biological species concept proposed by Dobzhansky (1937) and Mayr (1942). The difficulty persists because interpretation of the biological species concept suggests that complete reproductive isolation is a prerequisite for speciation, even though most species concepts, including the biological species concept, allow for some genetic exchange between species (Futuyma, 2005; Arnold, 2006). The requirement of reproductive isolation is especially problematic for conservation when species are part of a hybrid swarm, where introgressive hybridization with various levels of backcrossing has occurred between two or more species. Although these challenges could be alleviated by restructuring policies to focus on conserving evolutionary potential, identifying legitimate conservation units in hybrid populations under the current framework remains difficult.

Across Ontario, genomes from three different *Canis* species, grey wolves (*C. lupus*), eastern wolves (*C. lycaon*) and western coyotes (*C. latrans*), are represented in various hybridized forms, creating a three-species hybrid complex (Wilson *et al*., 2009; Wilson *et al*., in review) or syngameon. In contrast, grey wolves and coyotes in western North America, outside the historic range of eastern wolves (Rutledge *et al*., 2009), show no evidence of hybridization (Pilgrim *et al*., 1998). In fact, grey wolves aggressively limit coyotes, sometimes with fatal consequences (Berger and Gese, 2007; Merkle *et al*., 2009).

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It has been proposed that *Canis* hybridization flourished in eastern North America because of the presence of the eastern wolf, an intermediate-sized canid, that mediated gene flow between grey wolves and coyotes (Wheeldon and White, 2009; Wilson *et al*., 2009).

There has also been speculation that ecological factors may be promoting selection on morphological and genetic variation among contemporary *Canis* hybrid populations in Ontario (Wilson *et al*., 2009). Body size is notably different among the different regions with an increase in size along a latitudinal gradient, where animals in southern regions of Ontario are smaller than the intermediate-sized animals in Algonquin Provincial Park (APP), which in turn are smaller than those found in northern Ontario (Kolenosky and Standfield, 1975; Sears *et al*., 2003; Holloway, 2010; Figure 1). Presumably, selection is influenced by differences in prey availability (Carmichael *et al*., 2001; Muñoz-Fuentes *et al*., 2009) in the different regions, with larger ungulates such as moose (*Alces alces*) and woodland caribou (*Rangifer tarandus*) predominant in northern regions, intermediate-sized prey such as white-tailed deer (*Odocoileus virginianusvirginianus*) and beaver (*Castor canadensis*) being common along with moose in APP, and smaller prey in southern Ontario such as cottontail rabbit (*Sylvilagus floridanus*), groundhog (*Marmota monax*) and muskrat (*Ondatra zibethicus*), which exist sympatrically with abundant white-tailed deer populations. Moose have been a fluctuating presence in APP since the early 1900s (Quinn, 2005), but Algonquin wolves have typically preyed primarily on deer (Pimlott *et al*., 1969; Forbes and Theberge, 1996). Recent work, however, suggests that they may now be effective moose predators (Loveless, 2010). The regional prey base coincides with the broader landscape features of Ontario: northeastern Ontario (NEON) is primarily a remote boreal forest; APP is a protected area representing a zone that is transitional between deciduous forest south and coniferous forests north of the park; and southern Ontario is predominantly farmland with scattered deciduous forest patches and urban areas.

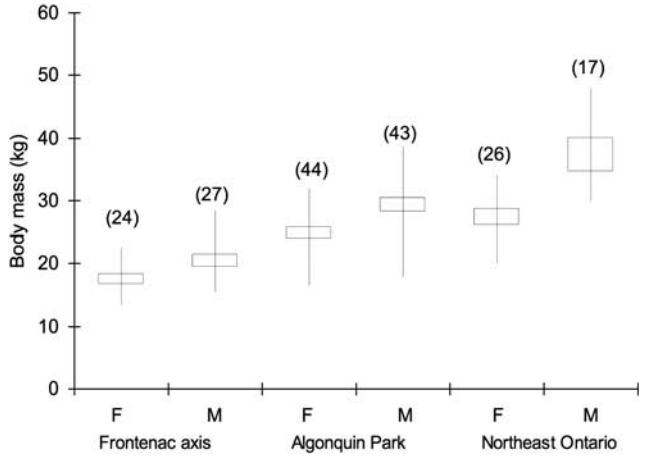

Figure 1 Relative weights (kg) of *Canis* hybrids from three regions of Ontario, Canada. Boxes represent the mean and 95% confidence intervals, whereas the whiskers indicate the range of values for each category. Sample sizes are given in parentheses. Weights from the Frontenac Axis were taken from Sears (1999), those from northeast Ontario were taken from Holloway (2010) and those from Algonquin Park were compiled from captures associated with pedigree analysis (Rutledge *et al*., 2010). M, male; F, female.

Characterizing the mechanisms, extent and timing (ancient, ~11 000 years ago; historic, approximately 60–100 years ago and/or ongoing) of *Canis* hybridization in eastern North America, and identifying post-hybridization differentiation are important for setting effective conservation policy in Canada and the United States. Previous research has suggested that eastern wolves in APP represent the southern extent of a larger metapopulation (Grewal *et al*., 2004; Wilson *et al*., 2009). However, the recent development of advanced analytical tools for genetic and spatial data, combined with pedigree data, allows for a more detailed account of gene flow and differentiation in Ontario *Canis* populations. To that end, we used maternal, paternal and biparental markers to generate genetic data on 217 *Canis* animals from three different geographic regions in Ontario: eastern coyotes (*C. latrans x lycaon*) from southern Ontario along the Frontenac Axis (FRAX), nominal eastern wolves (*C. lycaon* that have introgressed western coyote (*C. latrans*) and grey wolf (*C. lupus*) genetic material) from APP, and grey wolf hybrids (*C. lupus x lycaon*) from NEON (Figure 2). Although we acknowledge the hybrid nature of these animals, for simplicity we refer to these groups as coyotes (FRAX), eastern wolves (APP) and grey wolves (NEON) throughout the remainder of the paper. These data, combined with pedigree data from APP (Rutledge *et al*., 2010), were used to test the following hypotheses: (1) eastern wolves in APP have acted as a conduit of gene flow between grey wolves north of the park and coyotes south of the park; (2) hybridization events were historically gender biased with males of the larger species breeding with females of the smaller species and (3) eastern wolves in APP are a distinct genetic group despite ancient/historic hybridization with grey wolves and coyotes.

Materials and methods

Sample collection, DNA extraction and genetic profiling

Canis blood or tissue samples were collected and sample locations were recorded in three different geographic regions across Ontario: NEON ($n=51$), APP ($n=128$) and FRAX ($n=38$) (Figure 2). Samples from NEON were randomly selected from a data set of wolflike canids (Wheeldon, 2009). Based on assignment tests conducted with the software program Structure 2.2 (Pritchard *et al*., 2000; http://pritch.bsd.uchicago.edu/structure.html), only 3 of the 109 samples in a source NEON data set were highly assigned as eastern coyotes ($Q>0.8$), one was identified as an eastern wolf-coyote hybrid ($Q=0.453$; 0.477) and none were identified as a grey wolf–eastern coyote (NEON-FRAX) hybrid (based on eastern coyote $Q\geqslant 0.2$) (Wheeldon TJ, unpublished data). Animals from FRAX were randomly selected from a larger data set described in Sears *et al*. (2003). In APP, samples were chosen from a larger data set based on pack affiliations and pedigrees (Rutledge *et al*., 2010) such that only unrelated individuals within a pack and animals not affiliated with a pack were included. Pups and related yearlings were excluded from the analysis, except in cases where one or both the breeding pairs were not identified, in which case one pup was included

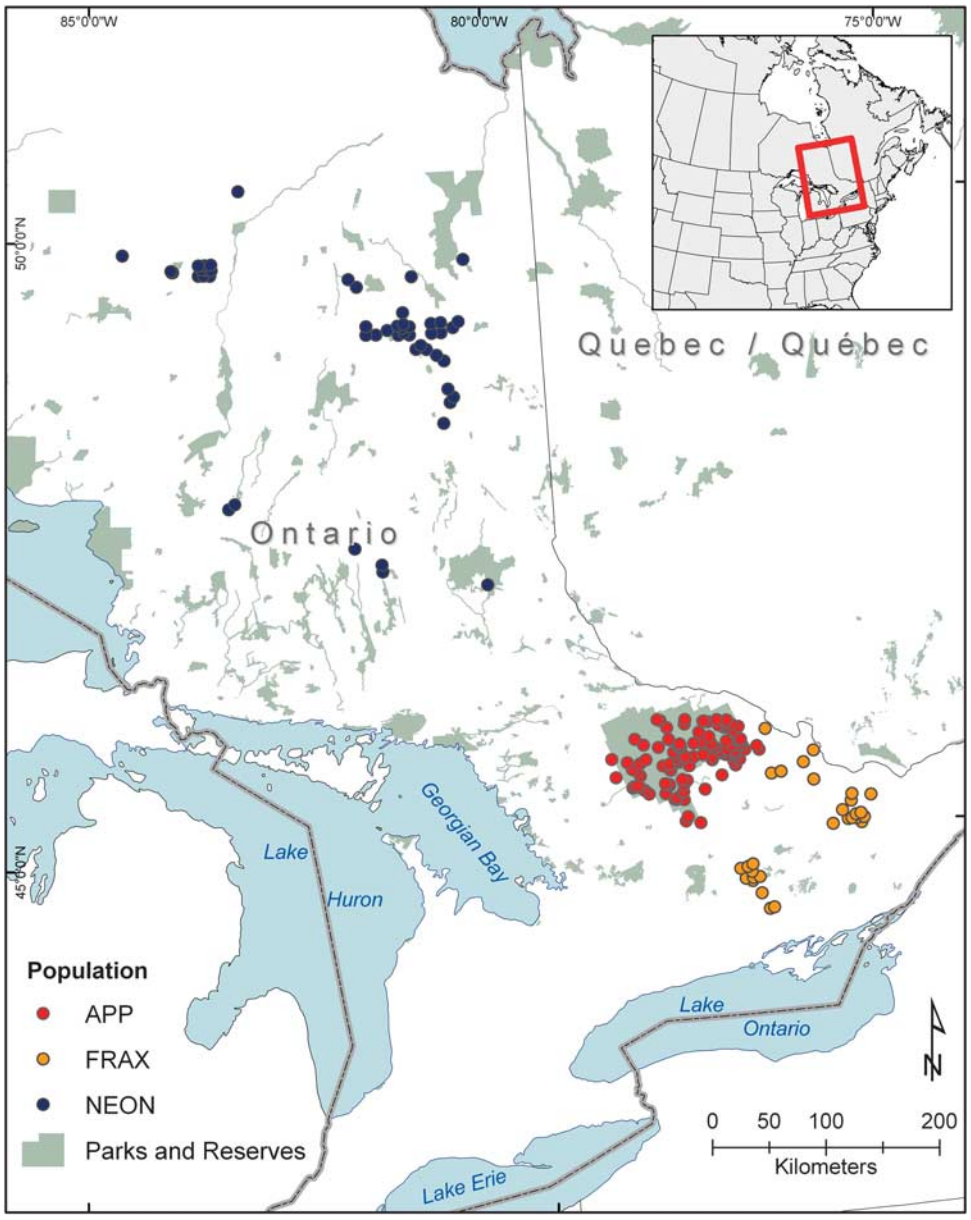

Figure 2 Map of the three sampling regions and locations of the *Canis* samples used in this study. APP, Algonquin Provincial Park; FRAX, Frontenac Axis in southern Ontario; NEON, northeastern Ontario.

as representative of the missing parental genome(s). On the basis of kinship analysis (Rutledge *et al*., 2010), animals identified as transient based on telemetry data were typically unrelated to each other. Although *Canis* spp. distribution is continuous between APP and NEON, samples from the region directly north of APP were unavailable. NEON and APP samples were extracted with a DNeasy Blood and Tissue kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's directions and those from FRAX were extracted as described in Grewal *et al*. (2004).

All samples were profiled at the mitochondrial DNA (mtDNA) control region with primers described in Wilson *et al*. (2000) and 12 autosomal microsatellite markers (cxx225, cxx200, cxx123, cxx377, cxx250, cxx204, cxx172, cxx109, cxx253, cxx442, cxx410 and cxx147; Ostrander *et al*., 1993, 1995) with conditions described in Wheeldon and White (2009). Gender was determined either by amplification at the Zfx/Zfy intron (Shaw *et al*., 2003) or with Zfx/Sry primer pairs (Aasen and Medrano, 1990; Fain and LeMay, 1995). Confirmed males were then profiled at 4 Y chromosome microsatellite loci (MS34A, MS34B, MS41A and MS41B; Sundqvist *et al*., 2001) and at a 658 bp fragment of the Zfy intron with primers LGL-331 (5′-CAAATCATGCAAGGATAGAC-3′; Shaw *et al*., 2003) and Yint2-335 (5′-GTCCATTGGATAATTCTTTCC-3′; Shami, 2002). The polymerase chain reaction (PCR) chemical and cycling conditions for the Y chromosome microsatellite loci were as follows: For MS34, 5–10 ng of DNA was amplified in a 15 µl reaction with 1 × PCR buffer, 0.2 mM dNTPs (Invitrogen, Burlington, Ontario, Canada), 1.5 mM $MgCl_2$, 0.1 µM MS34A-F primer, 0.15 µM MS34B-F primer, 0.2 µM MS34-R primer and 1 U *Taq* DNA polymerase (Invitrogen). PCR cycling included an initial

denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1:00, with a final cycle of 60 °C for 45 min and storage at 4 °C. Conditions for MS41 were similar to MS34, except that primer concentrations were 0.15 μM MS41A-F primer, 0.2 μM MS41B-F primer, 0.2 μM MS41-R primer and the annealing temperature was 58 °C. The Y intron was amplified under the following PCR conditions in a 20 μl reaction: approximately 5–10 ng of DNA, 1 × PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 mM each primer, 0.1 μg bovine serum albumin and 1 U *Taq* DNA polymerase. PCR steps included initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. All sequencing and microsatellite fragment separation and visualization were performed on a MegaBACE 1000 (G Healthcare, Baie d'Urfé, Quebec, Canada), with the exception of the NEON and FRAX Y chromosome microsatellites that were analysed on an AB3730 (Applied Biosystems Canada, Streetsville, Ontario, Canada). All microsatellites were scored in GeneMarker 1.7 (SoftGenetics, State College, PA, USA).

Sequences were edited in BioEdit (Hall, 2007) and mtDNA sequences were assigned unique haplotype codes (C*x*) identified in Wilson *et al*. (2000) and Grewal *et al*. (2004). Haplotypes C1 and C3 are considered eastern wolf specific based on previously published phylogenetic analyses (Wilson *et al*., 2000; Rutledge *et al*., 2009). Although haplotypes C9, C13 and C17 cluster phylogenetically with coyote haplotypes, leading some to interpret them as coyote specific (for example, Leonard and Wayne, 2008; Kays *et al*., 2010), they are interpreted here as eastern wolf specific because they are not known to occur in non-hybridized western coyote populations; their presence in eastern wolves is due to either incomplete lineage sorting or hybridization during the last glaciation event ~11 000 years ago (Wheeldon and White, 2009). This assumption is reasonable, given the presence of coyote-like haplotypes in eastern wolves approximately 30–400 years before western coyote expansion (Wilson *et al*., 2003; Rutledge *et al*., 2009), and the assignment of a coyote-like sequence as red wolf (*C. rufus*) specific (Hailer and Leonard, 2008).

Y intron sequences were identified as one of the four possible species-specific haplotypes (intron-1 (ancestral; GenBank accession no. FJ687618), intron-2 (grey wolf; GenBank accession no. FJ687619), intron-3 (coyote; accession number not available) or intron-4 (eastern wolf; GenBank accession no. FJ687620)) as described by Shami (2002) and Wilson *et al*. (in review). Y chromosome haplotypes associated with intron 1 are considered ancestral because introns 2, 3 and 4 each differ from intron 1 by only one nucleotide (Shami, 2002), although conservatively Intron 1 may be interpreted as a North American evolved Y chromosome haplotype that is shared between eastern wolves and coyotes (Wilson *et al*., in review). Y chromosome microsatellite haplotypes were assigned according to previously reported nomenclature (Grewal *et al*., 2004; Wilson *et al*., in review).

Genetic diversity and differentiation

Standard measures of genetic diversity in each of the three geographic regions were calculated in GenAlEx 6.1 (Peakall and Smouse, 2006). Traditional estimates of genetic differentiation based on F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) were calculated and tests of significance were performed with 999 permutations in the AMOVA option of GenAlEx 6.1 (Peakall and Smouse, 2006). However, F_{ST} is based on sample heterozygosity, and therefore differentiation can be underestimated when within-population heterozygosity is high or when heterozygosity varies among populations (Hedrick, 2005; Jost, 2008, 2009). In addition, R_{ST} assumes a stepwise mutation model for microsatellites that may not fully reflect how microsatellites evolve (Li *et al*., 2002). As we were interested in comparing differentiation among the three nominal species, we also calculated the harmonic mean of Jost's D_{est} (Jost, 2008) in the online program SMOGD version 1.2.5 (Crawford, 2009; http://www.ngcrawford.com/django/jost/; accessed 19 August 2009) as a measure of absolute differentiation between regions. The D_{est} measure is based on allele identities rather than ratios of heterozygosity.

Bayesian and multivariate ordination analyses

To determine genetic structuring and individual assignments based on the autosomal microsatellite data set, we used two different approaches: (1) Bayesian clustering assignments with and without spatial data implemented in the programs Geneland version 3.1.4 (Guillot *et al*., 2008) and Structure version 2.2 (Pritchard *et al*., 2000; Falush *et al*., 2003), respectively and (2) multivariate ordination methods with and without spatial components analysed by principal component analysis (PCA) and a spatial principal component analysis (sPCA) (Jombart *et al*., 2008), both implemented in the *adegenet* package (Jombart, 2008) of R 2.9.0 (R Development Core Team, 2008).

Using these methods in combination, one can assess the various assumptions and criticisms of each method when the combined results are interpreted. For example, Bayesian clustering programs such as Structure provide powerful analytical tools for identifying genetic structure in data sets, and similar programs such as Geneland incorporate spatial data to assess the influence that geography has on population structure (Coulon *et al*., 2006). However, both approaches assume that populations are in the Hardy–Weinberg equilibrium and that there is linkage equilibrium between loci, prerequisites that are often violated in natural populations. In contrast, multivariate ordination in a PCA does not require the data to meet those assumptions and thereby complements the Bayesian analyses. Recently, Jombart *et al*. (2008) suggested that Bayesian clustering may be inappropriate when populations are structured across a cline, and they developed a 'spatially explicit multivariate method' or sPCA that accounted for spatial structure and genetic variability and could identify different genetic structures, including clines, without having to meet the assumptions of Bayesian approaches. The sPCA complements the Bayesian approach implemented in Geneland by identifying more cryptic spatial patterns of genetic structuring across the landscape, and accounts for spatial autocorrelation issues associated with neighbour-mating and sample distribution (Schwartz and McKelvey, 2009).

The number of clusters (K) was estimated in Structure version 2.2 with no *a priori* population assignment of individuals under the admixture F model for correlated allele frequencies (Falush *et al*., 2003) with 5 000 000 MCMC steps and a burn-in of 250 000. Five runs, each of $K = 1$–8, were used to determine the most likely number of clusters based on a combination of the mean estimated Ln probability of the data (Pritchard *et al*., 2000) and the second-order rate of change in the log probability of the data (ΔK) (Evanno *et al*., 2005). A total of 10 runs at $K = 3$ were subsequently run under the same conditions and average Q scores were used as the probability of assignment. Values of $Q \geqslant 0.8$ were considered 'pure' on the basis of standard assumptions in the literature and previous hybrid simulation analyses performed in R with a similar data set (L Rutledge, unpublished data). Individuals were considered 'admixed' if there was no single assignment of $Q > 0.8$ and at least one secondary probability value was $Q \geqslant 0.1$.

The Geneland algorithm is similar to Structure in that it attempts to define clusters by maximizing Hardy–Weinberg equilibrium and linkage equilibrium. It differs in that it treats K as a parameter to be estimated by the MCMC algorithm, and it can explicitly incorporate the spatial coordinates of individuals into its modelling procedure, which may be particularly important when examining spatial processes such as gene flow. Parameters were set as follows: 1 000 000 MCMC iterations, maximum rate of Poisson process was 100, and the uncertainty of spatial coordinates was 1 km. With these parameters, the MCMC was run 10 times, allowing K to vary between 1 and 10 to verify the consistency of the inferred K (Guillot *et al*., 2005). The MCMC was then run 25 more times with the same parameters but with K fixed to the value inferred by the initial run. From this set, the run with the highest log posterior probability of population membership was selected for subsequent analyses. The posterior probability of population membership for pixels was computed with a burn-in of 500 000 iterations and the number of pixels was set to 300 along both the x and y axes. Finally, the posterior probability of population membership was computed for pixels and the inferred population membership of individuals to model populations.

A centred, scaled PCA was used to cluster individual microsatellite genotypes. We chose to use PCA because it clusters individuals only on the basis of their genotypes and makes no assumptions regarding Hardy–Weinberg equilibrium or linkage equilibrium. Thus, it is a good option to corroborate inferences from the Structure analysis while making fewer assumptions regarding the underlying data structure. The PCA was implemented in the R statistical language (R Development Core Team, 2008) with the *adegenet* (Jombart, 2008) and *ade4* (Dray and Dufour, 2007) packages.

As noted by the authors of the Bayesian algorithms used here (Pritchard *et al*., 2000; Guillot *et al*., 2005) and recently shown by simulation (Frantz *et al*., 2009; Schwartz and McKelvey, 2009), deviations from random mating not caused by barriers to gene flow (that is, spatial autocorrelation and isolation by distance) and the sampling scheme can have impacts on the detection and interpretation of genetic structure. These include potential overestimation of genetic structure for data sets characterized by continuously distributed individuals and spatially autocorrelated allele frequencies (Frantz *et al*., 2009; Schwartz and McKelvey, 2009). sPCA (Jombart *et al*., 2008) explicitly incorporates spatial autocorrelation as well as variance into the clustering procedure. Similar to PCA, sPCA makes no assumptions regarding Hardy–Weinberg equilibrium or linkage equilibrium and thus provides a useful corroboration to the similarly spatial Geneland while making fewer assumptions. sPCA has another important feature that differs from the Structure, PCA and Geneland analyses: it can explicitly identify spatial clines. sPCA incorporates Moran's I (Moran, 1948, 1950) to detect spatial features in the data. A Gabriel graph (Legendre and Legendre, 1998) was constructed based upon individual sample locations to define neighbours for the calculation of Moran's I. sPCA then defines synthetic components that optimize the product of the variance and Moran's I, summarizing spatial patterns of genetic structure. These components are separated into positive (global) and negative (local) eigenvalues. Global scores can identify genetically distinguishable groups, clines in allele frequencies and intermediate individuals, whereas local scores can detect differentiation between neighbouring individuals. As with the PCA, the sPCA analysis was implemented in R with the *adegenet* (Jombart, 2008) and *ade4* (Dray and Dufour, 2007) packages. Principal component values were interpolated as a function of x and y coordinates using a local least-squares regression and plotted with the 's.image' function in the *ade4* package (Dray and Dufour, 2007) on a map of Ontario.

Mating patterns

To determine patterns of sex-biased hybridization events, we compiled mtDNA and Y chromosome haplotypes for all individuals. For identification of conspecific mating, breeders and breeding pairs (which tended to be unrelated) within the APP data set were identified based on previous pedigree analysis (Rutledge *et al*., 2010). Admixed individuals were identified as described in the Structure methods above. Both the assignment scores in Structure and the mtDNA and Y chromosome DNA haplotypes were identified for all APP breeders. To test whether mating patterns in APP were assortative for (1) the APP population, and (2) the data set as a whole, we used randomization tests (100 permutations) on the Y chromosome microsatellite haplotype data to determine whether the observed proportion of male breeders with eastern wolf Y chromosome haplotypes in APP differed from that expected if mating within APP were random. Based on the identification of 18 breeding males in APP, resampling of 18 Y chromosome microsatellite haplotypes from those identified in all males (1) within APP and (2) across all regions was performed without replacement under the assumption that male wolves typically father offspring within only one pack.

Results

Genetic diversity and differentiation

Genetic diversity was high in all three geographic regions and each group showed evidence of private alleles (Table 1). Pairwise comparisons of genetic differentiation showed significant differentiation between groups, although R_{ST} values suggest a closer

Table 1 Genetic diversity within *Canis* clusters

Cluster	N	A *(s.e.)*	A_E *(s.e.)*	H_O *(s.e.)*	H_E *(s.e.)*	A_P
APP	128	7.58 (0.657)	3.561 (0.333)	0.645 (0.041)	0.678 (0.043)	4
FRAX	38	6.83 (0.694)	4.281 (0.408)	0.717 (0.042)	0.733 (0.035)	4
NEON	51	6.33 (0.497)	3.629 (0.496)	0.688 (0.037)	0.678 (0.033)	5

Abbreviations: *A*, average number of alleles; A_E, effective number of alleles; A_P, number of private alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *N*, samples sizes.
Standard error (s.e.) is shown in parentheses.

Table 2 Pairwise comparisons of F_{ST} (Weir and Cockerham, 1984), R_{ST} (Slatkin, 1995), and D_{est} (Jost, 2008) among *Canis* clusters

Measure	*APP-FRAX*	*APP-NEON*	*NEON-FRAX*
F_{st}	0.052 (0.001)	0.105 (0.001)	0.120 (0.001)
R_{st}	0.029 (0.007)	0.008 (0.06)	0.194 (0.001)
D_{est}	0.090	0.207	0.255

For F_{ST} and R_{ST}, numbers in parentheses are *P*-values based on 999 permuations in the AMOVA option of GenAlEx 6.1 (Peakall and Smouse, 2006). D_{est} is the harmonic mean across loci as calculated in SMOGD (Crawford, 2009).

relationship between APP and NEON than between APP and FRAX, whereas F_{ST} and D_{est} show APP as more closely related to FRAX than NEON (Table 2).

Bayesian and multivariate ordination analyses

Bayesian clustering with Structure and Geneland converged on three groups (Figures 3a and 4a). The high peak for ΔK at $K=2$ is interpreted as the broad separation 1–2 million years ago between the Old World (grey wolves) and New World (eastern wolves/red wolves and coyotes) lineages. Concordance between both estimation methods in Structure is found at $K=3$, which may reflect the more recent divergence among the New World species (approximately 150 000–300 000 years ago) (Wilson *et al*., 2000). Although there is clear differentiation among the three geographic regions and posterior probabilities in Geneland showed a strong association to each geographic region with a steep division between APP and FRAX (Figures 3b and 4a–d), there is evidence of some admixture and migration (Figure 3b). Both FRAX ($n=6$; 15.8%) and NEON ($n=8$; 15.7%) had animals admixed with APP, and APP had evidence of admixture with both FRAX ($n=14$; 10.9%) and NEON ($n=8$; 6.3%). Only two animals from NEON had evidence of FRAX hybridization, but both had $Q_{FRAX}<0.3$ (Figure 3b). Two animals were migrants from APP into FRAX ($Q_{APP}=0.9821$; 0.9248) and three migrated from FRAX to APP ($Q_{FRAX}=0.9085$; 0.9094; 0.8245), but no migrants were identified between FRAX and NEON (although known coyotes were excluded from the NEON data set because we were interested in sampling wolflike animals in this region; there was no evidence of NEON–FRAX hybridization in the original data set; see Materials and methods section for more details).

Similar to the Bayesian analyses, components 1 and 2 of the PCA clearly differentiated APP, NEON and FRAX, with APP intermediate to the other clusters but more closely associated with FRAX, although there is evidence of migration between the two groups (Figure 5). In the

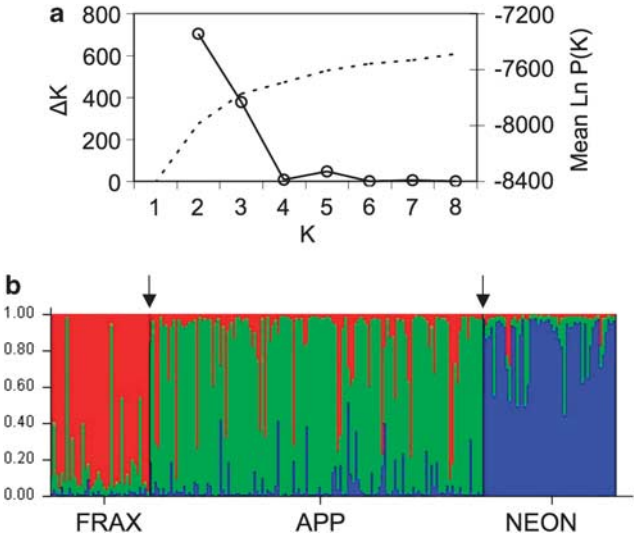

Figure 3 (**a**) Estimation of the number of *Canis* clusters (*K*). Dotted line is the mean Ln probability of the data (Ln *P*(*K*)) (Pritchard *et al*., 2000) and the solid line is the second-order rate of change (ΔK) (Evanno *et al*., 2005), inferring that $K=3$. (**b**) Bayesian clustering and individual assignment output from the program Structure show genotypic clustering according to geographic regions with some evidence of admixture within each cluster. Arrows indicate divisions of geographic regions. Vertical axis is the probability of assignment (*Q*) to each of the clusters, and the horizontal axis represents the categories of geographic regions sampled: APP, Algonquin Provincial Park; FRAX, Frontenac Axis in southern Ontario; NEON, northeastern Ontario.

sPCA, three global components appeared to be important. Interpolation using a locally weighted regression (LOESS) of component 1 scores from the sPCA illustrated a cline in allele frequencies from south to north (Figure 6), supporting the contention that eastern wolves in APP act as a conduit for gene flow between coyotes in the FRAX regions and grey wolves in the NEON region. Subsequent sPCA components clustered the populations similar to the other clustering methods (data not shown).

Mating patterns

Patterns of mtDNA and Y chromosome haplotype distributions across the three geographic regions were generally consistent with the hypothesis that introgression was directional with females of the smaller species historically mating with males of the larger species (Tables 3 and 4). Sex-biased introgression was clearly evident when comparing FRAX and NEON haplotypes, but these patterns were less clearly defined when APP was compared with the other two groups (Tables 3 and 4). Grey wolf mtDNA was common in

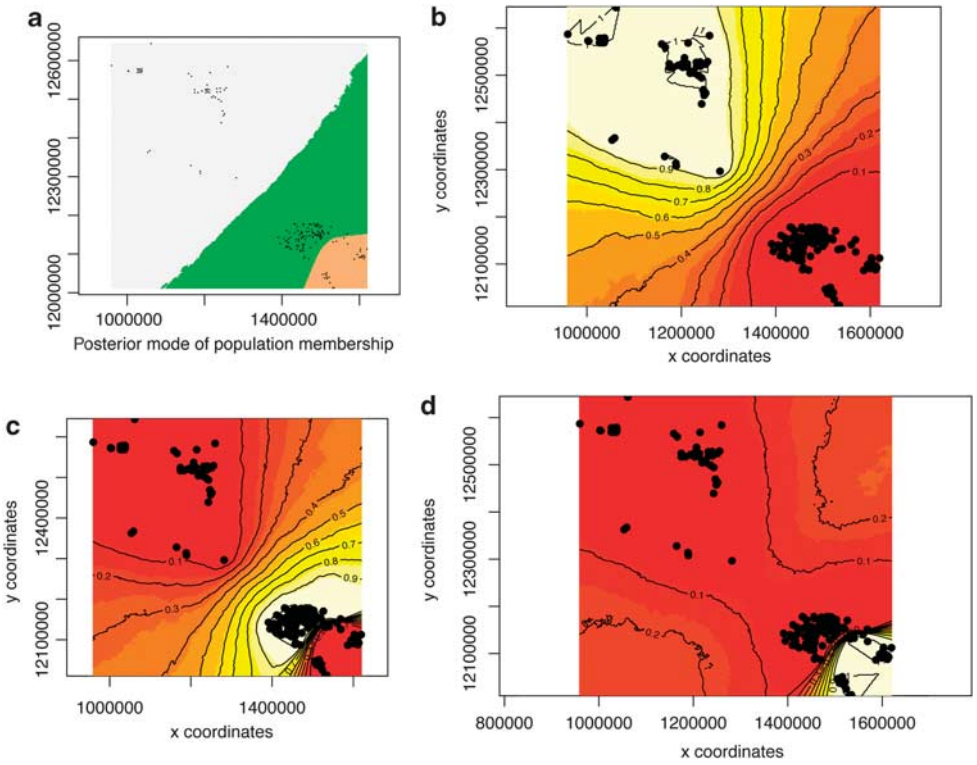

Figure 4 Spatial Bayesian clustering in the program Geneland. Black dots represent individual *Canis* samples. (**a**) Spatial clustering suggests three distinct clusters across geographic regions; (**b**) map of posterior probability of belonging to the northeastern Ontario (NEON) cluster; (**c**) map of the posterior probability of belonging to the Algonquin Provincial Park (APP) cluster; (**d**) map of the posterior probability of belonging to the southern Frontenac Axis (FRAX) cluster.

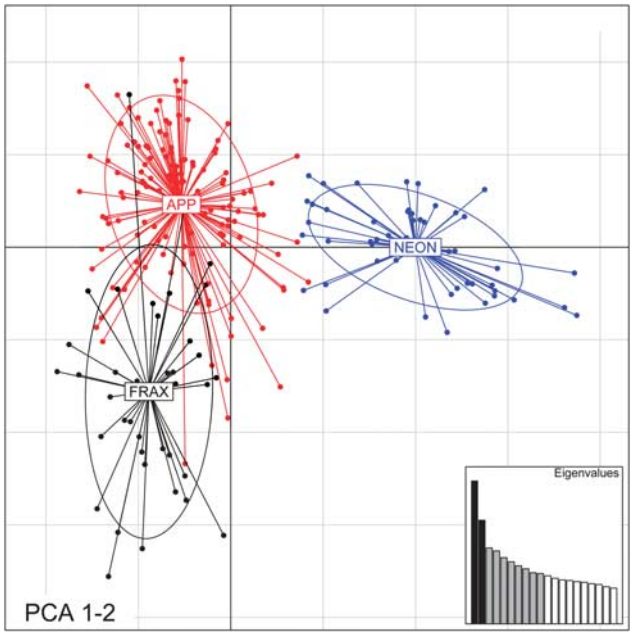

Figure 5 First and second components of a principal components analysis (PCA) of 12-locus microsatellite genotypes from 217 *Canis* samples from Ontario, Canada. APP, Algonquin Provincial Park; NEON, northeastern Ontario; FRAX, Frontenac Axis in southern Ontario. Ovals are 95% inertia ellipses.

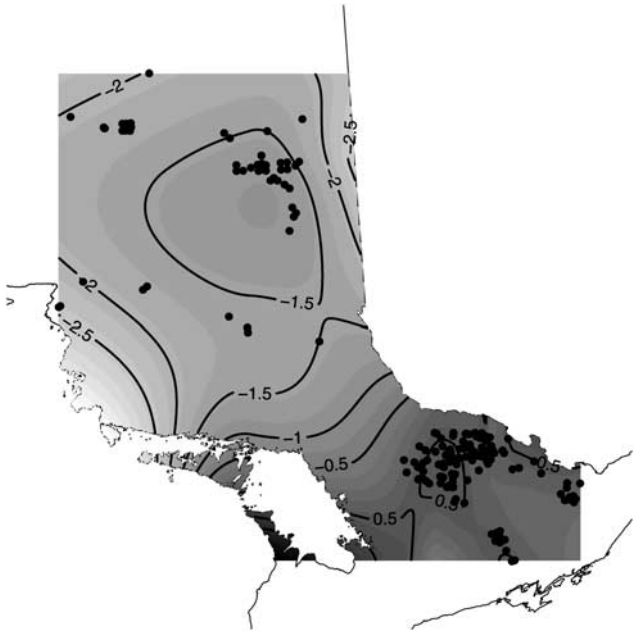

Figure 6 Component 1 from a spatial principal components analysis (sPCA) of 12-locus microsatellite genotypes from 217 *Canis* samples from Ontario, Canada, plotted on a map of Ontario. Dots are sample locations and contours are component scores that represent similarity across the landscape.

NEON with some evidence in APP but was absent from FRAX (Table 3). There were no coyote-specific Y introns found in any of the regions, although APP and FRAX had Y introns and Y microsatellite haplotypes identified as ancestral haplotypes that evolved in North America (Shami, 2002; Wilson *et al*., in review; Table 4). This

ancestral Y chromosome haplotype was absent from NEON. There was evidence of eastern wolf-specific mtDNA and Y chromosome DNA in all three groups, although only one eastern wolf Y chromosome haplotype was found in NEON (Table 4).

MtDNA haplotypes, Y chromosome haplotypes and assignment scores of all breeders identified in APP are shown in Table 5. Breeding wolves in APP tended to have high assignment to the Algonquin cluster and paired breeders were generally conspecific based on nuclear markers (Table 5). Of the 19 female breeders, 11 (57.9%) had coyoto mtDNA haplotypes (C14, C19), 2 (10.5%) had grey wolf mtDNA (C22) and 6 (31.6%) had eastern wolf haplotypes (C9, C17) (Table 5). Twenty-nine of the 37 breeders (78.4%) and 11 of the 15 breeding pairs (73.3%) had high assignment scores ($Q \geqslant 0.8$) to the APP cluster, whereas the remaining individuals or breeding pairs had varying levels of admixture between two or among all three clusters (Table 5). Of the 18 breeding males, 16 (88.9%) had an eastern wolf Y chromosome haplotype (4AA, 4BB) with the other two having a grey wolf haplotype (2EF, 2CS); no male breeders had an ancestral or coyote Y chromosome haplotype (Table 5). Of all the male breeders, only those two with a grey wolf Y chromosome haplotype were identified as admixed individuals. The proportion of eastern wolf Y chromosome haplotypes in APP breeding males was significantly higher than that expected from random mating across the complete Y chromosome microsatellite data set ($P < 0.01$) and that within APP ($P < 0.02$; Figure 7).

Table 3 Mitochondrial DNA control region haplotypes in the three *Canis* clusters

Haplotype	*GenBank accession no.*	*FRAX* (n)	*APP* (n)	*NEON* (n)	*Origin*
C14	AY267731	6	65	17	Coyote
C19	AY267736	6	33	0	Coyote
C20	AY267737	3	0	0	Coyote
C1	AY267718	9	3	0	Eastern
C3	AY267720	0	1	1	Eastern
C9	AY267726	14	7	0	Eastern
C13	AY267730	0	1	6	Eastern
C17	AY267734	0	8	0	Eastern
C22	FJ687608	0	9	17	Grey
C23	FJ687609	0	0	9	Grey
C95	FJ687610	0	0	1	Grey
Missing		0	1	0	

Discussion

Our study results clearly show that the three *Canis* populations in Ontario are genetically distinct, despite evidence of some contemporary gene flow. Current *Canis* species in North America diverged relatively recently, all sharing a common ancestor approximately 1–2 million years ago (Wilson *et al*., 2000), which may facilitate their successful mating when secondary contact occurs. Accordingly, the intermediate-sized wolves in APP contain a patchwork of genetic material derived from eastern wolves (*C. lycaon*), western coyotes (*C. latrans*) and grey wolves (*C. lupus*), having acted as a bridge for genetic exchange between grey wolves and coyotes. Animals in APP have, however, emerged as a distinct cohesive genetic unit despite hybridization with the other species. Under the framework of the Cohesion Species Concept whereby 'A species is the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms' (Templeton, 1989), eastern wolves in APP can be defined as a separate species. Given the assortative mating patterns identified here, the mechanism by which these animals remain distinct may involve a Specific-Mate-Recognition System whereby animals are able to recognize conspecifics as mates on the basis of behavioural, chemical or visual signals (Paterson, 1985; Coyne and Orr, 2004). The mechanism by which this occurs in wolves is not clear, but could presumably involve any one or all of these cues.

Patterns of maternal (mtDNA) and paternal (Y chromosome) inheritance indicate that introgression was, in general, sex biased with maternal DNA from the smaller coyote and paternal DNA from the larger grey wolf flowing across the landscape through eastern wolves. The presence of grey wolf mtDNA in APP and eastern wolf mtDNA in FRAX can be explained by introgression through hybrid backcrossing. Prevalence of eastern wolf mtDNA across the eastern United States

Table 4 Y chromosome haplotypes in the three *Canis* clusters

Hap	*Intron*	*MS34a*	*MS34b*	*MS41a*	*MS41b*	*FRAX* (n)	*APP* (n)	*NEON* (n)	*Origin*
CD	1	172	178	214	210	4	2	0	A
CR	1	172	178	212	216	0	1	0	A
CI	1	172	178	214	214	1	0	0	A
GP	1	176	180	212	222	1	1	0	A
AA	4	172	180	212	212	9	26	0	EW
BB	4	170	182	212	226	0	14	1	EW
EF	2	176	178	208	222	0	3	0	GW
AF	2	172	180	208	222	0	0	4	GW
FL	2	174	178	208	218	1	0	0	GW
HS	2	174	176	208	226	1	0	0	GW
HT	2	174	176	208	220	1	0	0	GW
CS	2	172	178	208	226	0	3	0	GW
CE	2	172	178	208	216	1	2	8	GW
CF	2	172	178	208	222	0	0	5	GW
CT	2	172	178	208	220	0	0	8	GW
CG	2	172	178	208	224	0	1	0	GW

Abbreviations: A, ancestral; EW, eastern wolf; GW, grey wolf; Hap, haplotype.

Table 5 Assignment scores (*Q*) of known breeders in APP wolf packs

Individual ID	*Gender*	Q_{APP}	Q_{FRAX}	Q_{NEON}	*mtDNA haplotype*	*Y chromosome haplotype*
Achray						
W89/C4337	M	**0.9621**	0.0206	0.017	C14	4AA
W14/C4262	F	**0.9567**	0.0221	0.021	C17	NA
W131/C4379	M	**0.9687**	0.021	0.01	C14	4AA
W44/C4292	M	**0.975**	0.009	0.0168	C14	4AA
Beechnut						
W119/C4367	M	**0.1866**	**0.2981**	**0.5153**	C14	2EF
W130/C4378	F	**0.843**	0.0275	0.1295	C14	NA
Bena						
W21/C4269	M	**0.8612**	0.074	0.0648	C14	4AA
W42/C4290	F	**0.8517**	0.1383	0.01	C19	NA
Big crow						
W207/C4455	M	**0.9514**	0.0232	0.025	C14	4BB
W76/C4324	F	**0.968**	0.0211	0.011	C14	NA
Cauliflower						
W49/C4297	F	**0.8054**	0.1846	0.01	C19	NA
W122/C4370	F	**0.5678**	**0.3543**	0.0778	C19	NA
W16/C4264	F	**0.9557**	0.0221	0.022	C19	NA
Flat iron						
W58/C4306	F	**0.948**	0.0139	0.0383	C17	NA
Jocko						
W46/C4294	M	**0.966**	0.0236	0.0101	C14	4AA
W2/C4250	M	**0.948**	0.0311	0.0203	C14	4AA
W200/C4448	F	**0.976**	0.011	0.013	C22	NA
LaFleur						
W1/C4249	F	**0.6474**	**0.1614**	**0.1911**	C9	NA
W4/C4252	F	**0.2125**	**0.7398**	0.0478	C9	NA
Leaf						
W13/C4261	F	**0.7466**	0.0659	**0.1875**	C19	NA
W37/C4285	F	**0.2878**	**0.2875**	**0.4248**	C19	NA
W68/C4316	M	**0.9778**	0.0163	0.006	C19	4AA
Louisa						
W7/C4255	M	**0.9387**	0.0531	0.008	C19	4AA
W12/C4260	F	**0.6081**	**0.369**	0.023	C19	NA
McKaskill						
W50/C4298	M	**0.7677**	0.0954	**0.137**	C14	2CS
Pine						
W91/C4339	M	**0.973**	0.015	0.013	C17	4AA
W45/C4293	F	**0.9401**	0.0475	0.012	C14	NA
Potter						
W90/C4338	M	**0.9666**	0.013	0.02	C14	4BB
W190/C4438	M	**0.9527**	0.0279	0.0197	C14	4BB
Pretty						
W65/C4313	M	**0.983**	0.01	0.006	C17	4AA
W66/C4314	F	**0.978**	0.012	0.01	C22	NA
Radiant						
W84/C4332	M	**0.980**	0.009	0.011	C14	4AA
W62/C4310	F	**0.9102**	0.0267	0.0632	C14	NA
Spoor						
W125/C4373	M	**0.9606**	0.01	0.0294	C22	4AA
W124/C4372	F	**0.8813**	0.0259	0.0928	C19	NA
Sunday						
W180/C4428	M	**0.9209**	0.026	0.053	C19	4BB
W25/C4273	F	**0.9455**	0.0441	0.01	C14	NA

Abbreviations: F, female; M, male; NA, not applicable.
Pack names are given at the centre top of each group. Bold IDs show breeding pairs that produced offspring. Assignment scores are the probability values based on output from the program Structure and those in bold highlight either high assignment to APP ($Q \geqslant 0.8$) or various levels of admixture. Species designations of mtDNA and Y chromosome hapltoypes are indicated in Tables 3 and 4, respectively.

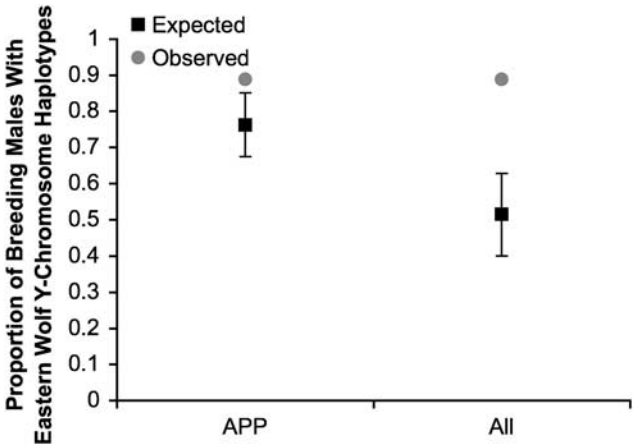

Figure 7 Observed proportion of eastern wolf Y chromosome haplotypes in Algonquin Provincial Park (APP) compared with that expected if males were breeding randomly. Randomization tests were conducted by randomly selecting 18 Y chromosome haplotypes from all possible Y chromosome haplotypes within APP ($P<0.02$) and across all populations ($P<0.01$). Expected values are based on 100 permutations. Error bars are the standard deviation.

(Kays *et al*., 2010; Way *et al*., 2010) suggests that the size hypothesis may be less applicable in the case of eastern wolf–coyote hybridization, but does not preclude the general trend observed in our data set where coyote mtDNA was present in NEON grey wolves but coyote and ancestral Y chromosome DNA was absent, and grey wolf mtDNA was present in FRAX coyotes but grey wolf Y chromosome DNA was absent.

The Bayesian and multivariate analyses used in this study revealed a complex history of repeated hybridization events in Ontario *Canis* populations. Our data support previous findings that show three genetic clusters in Ontario segregating as the near north Ontario grey wolf (also known as the 'boreal-type wolf', 'Ontario-type wolf' (Kolenosky and Standfield, 1975), or 'Great Lakes wolf' (Leonard and Wayne, 2008)), the APP eastern wolf and the eastern coyote (also referred to as the 'Tweed-type wolf' (Kolensoky and Standfield, 1975)) with some evidence of admixture, migration and a genetic cline across a latitudinal gradient; genetic material from *C. lycaon* is persistent across study regions. Overall, however, ongoing introgression is limited, possibly because of reproductive barriers in Algonquin animals that limit genetic exchange with neighbouring populations. Concentric sampling in areas outside the park, including Quebec, will be required to identify the full extent of the population of Algonquin-like canids.

Our interpretation is different from previous suggestions that Algonquin animals represent the southern edge of a larger metapopulation (Grewal *et al*., 2004; Wilson *et al*., 2009), but is consistent with morphological and ecological data that support the Algonquin animals as distinct from surrounding populations (Kolenosky and Standfield, 1975; Theberge and Theberge, 2004; Holloway, 2010). Although results from both the current and previous studies suggest gene flow across a cline, homogeneous neighbouring populations are not evident; the three *Canis* groups remain quite distinct. This apparent contradiction is illusory, however, as genetic exchange does not preclude differentiation, and evidence of gene flow at neutral markers is not necessarily indicative of sharing of coding regions responsible for phenotypic traits that are selected for in 'ecologically specialized populations' (Via, 2009). Future work that focuses on quantitative trait loci for morphological and behavioural features in *Canis* species, in conjunction with specific measurements of ecological variables, could be helpful in further understanding the role that divergent selection is having on *Canis* speciation. Differentiation in wolves is most likely linked to prey specialization (Carmichael *et al*., 2001; Muñoz-Fuentes *et al*., 2009). Therefore, if moose persists in APP, selection may favour larger eastern wolves either through natural selection on current morphological variation or through increased hybridization with larger grey wolves north of the park. Alternatively, transmission of learned behaviour through generations may be sufficient to allow wolves in Algonquin to exploit this large-bodied prey despite their relatively small stature. Regardless of the mechanism, protecting connected habitats will be important for gene flow into the park to maximize standing genetic variation on which selection can act.

We have documented a tendency for conspecific mating within the Algonquin wolf population. Assortative mating has a particularly important role in speciation because it is one of the main factors affecting ecological speciation events (Coyne and Orr, 2004; Schluter, 2009). Initially, divergent selection can influence reproductive fitness if hybrids are less fit than parental types (Schluter, 2001). Conspecific mate choice may provide a mechanism by which Algonquin animals are able to exclude hybrid species in neighbouring regions. Identifying the specific sensory cues involved in mate choice would help clarify how wolves identify conspecifics and what role that may have in ecological speciation.

In broad evolutionary terms, these results provide important insight into how mating patterns influence differentiation after hybridization. More specifically, however, the results of our study have important conservation implications for wild *Canis* species in eastern North America. The primary goal of conservation science is to conserve the genetic building blocks within species so that the evolutionary processes that contribute to ecological viability are minimally constrained. Eastern wolves are currently identified as a species of 'Special Concern' under the Committee on the Status of Endangered Wildlife in Canada and the Committee on the Status of Species at Risk in Ontario, but hybridization has complicated efforts to identify distinctive populations. The data presented here suggest that wolves in APP are morphologically and genetically distinct from *Canis* hybrids in other regions of Ontario, and contain a substantial proportion of the historic eastern wolf genome. Although range delineation of the APP population may be complicated by ongoing gene flow, this population constitutes a substantive, rare and likely ecologically diverged entity, and as such should be treated as a distinct conservation unit.

Conflict of interest

The authors declare no conflict of interest.

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