

Sympatric wolf and coyote populations of the western Great Lakes region are reproductively isolated

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Abstract

Interpretation of the genetic composition and taxonomic history of wolves in the western Great Lakes region (WGLR) of the United States has long been debated and has become more important to their conservation given the recent changes in their status under the Endangered Species Act. Currently, the two competing hypotheses on WGLR wolves are that they resulted from hybridization between (i) grey wolves (*Canis lupus*) and western coyotes (*C. latrans*) or (ii) between grey wolves and eastern wolves (*C. lycaon*). We performed a genetic analysis of sympatric wolves and coyotes from the region to assess the degree of reproductive isolation between them and to clarify the taxonomic status of WGLR wolves. Based on data from maternal, paternal and bi-parental genetic markers, we demonstrate a clear genetic distinction between sympatric wolves and coyotes and conclude that they are reproductively isolated and that wolf–coyote hybridization in the WGLR is uncommon. The data reject the hypothesis that wolves in the WGLR derive from hybridization between grey wolves and western coyotes, and we conclude that the extant WGLR wolf population is derived from hybridization between grey wolves and eastern wolves. Grey-eastern wolf hybrids (*C. lupus* × *lycaon*) comprise a substantial population that extends across Michigan, Wisconsin, Minnesota and western Ontario. These findings have important implications for the conservation and management of wolves in North America, specifically concerning the overestimation of grey wolf numbers in the United States and the need to address policies for hybrids.

Keywords: *Canis*, hybridization, microsatellite genotype, mitochondrial haplotype, Y chromosome

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Introduction

There has been longstanding debate concerning the taxonomic status of *Canis* populations in the Great Lakes region of North America, where interbreeding between species has led to a gradient of genetic composition and morphological forms (Kolenosky & Standfield 1975; Wayne & Vila 2003; Kyle *et al.* 2006; Wilson *et al.* 2009). Previous taxonomic designations for *Canis* were largely based on morphological differences and phenotypic variation (Pocock 1935; Goldman 1944; Nowak 1979), but more recently molecular genetics studies have

helped to more clearly elucidate their evolutionary history and taxonomy. It is generally accepted that the progenitor of the grey wolf (*Canis lupus*) migrated from North America to Eurasia and evolved there approximately 1–2 million years ago and later returned as the grey wolf (Nowak 1979; Lehman *et al.* 1991; Wilson *et al.* 2000). Based on genetic evidence, the eastern wolf (*C. lycaon*) was suggested to be a distinct species, conspecific to the red wolf (*C. rufus*), that evolved in North America sharing a common lineage with the western coyote (*C. latrans*) until diverging approximately 150 000–300 000 years ago (Wilson *et al.* 2000). Although alternate hypotheses exist regarding the origin of the eastern wolf, an extensive review of the molecular data (Kyle *et al.* 2006) supported the assessment of Wilson

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et al. (2000), rejecting the hypotheses that the eastern wolf is a grey wolf subspecies or the result of hybridization between grey wolves and coyotes. Furthermore, supporting data in the literature on the eastern wolf continues to accumulate (e.g. Wheeldon & White 2009; Wilson *et al.* 2009; Fain *et al.* 2010; Mech 2010; Way *et al.* 2010).

The colonization of northeastern North America by western coyotes in the last century resulted in hybridization with eastern wolves that led to the formation of the eastern coyote (*Canis latrans* × *lycaon*) (Hilton 1978; Schmitz & Kolenosky 1985; Wilson *et al.* 2009; Kays *et al.* 2010; Way *et al.* 2010). In contrast, no direct hybridization has been confirmed between grey wolves and coyotes in populations west of the Great Lakes region (Pilgrim *et al.* 1998; Hailer & Leonard 2008). However, there is evidence that eastern wolves and grey wolves have hybridized (Mech & Federoff 2002; Wheeldon & White 2009; Wilson *et al.* 2009; Fain *et al.* 2010). Agricultural and forestry practices along with predator control programs may have contributed to the breakdown of reproductive barriers between *Canis* species, facilitating their hybridization, as previously suggested (Kolenosky & Standfield 1975; Kyle *et al.* 2006).

The presence of 'coyote-clade' mtDNA in wolves from northeastern North America prior to the colonization of the region by western coyotes (Wilson *et al.* 2003; Leonard & Wayne 2008; Rutledge *et al.* 2009) suggests that historic hybridization may have occurred between eastern wolves and coyotes. Incomplete lineage sorting is likely for eastern wolves and coyotes given their close evolutionary relationship, probable historic hybridization, and demonstrated contemporary hybridization, thus reciprocal monophyly would not be expected for mtDNA phylogenies. Accordingly, gene trees based on nonrecombining markers, such as mtDNA and the Y chromosome, may be inadequate or unreliable for differentiating between the genetic contributions of coyotes and eastern wolves. This logic follows the caution, noted by Mech (2010), that gene trees may not necessarily represent species trees, which is apparent for eastern wolves and coyotes (Wilson *et al.* 2003; Leonard & Wayne 2008; Rutledge *et al.* 2009; Fain *et al.* 2010). For studies of hybridizing *Canis*, describing gene flow should be a useful approach for distinguishing between historic and contemporary hybridization. This approach requires genetic data from recombining markers and information on the geographical distribution of haplotypes and their temporal presence or absence in the studied populations.

By 1960, wolf populations were reportedly extirpated from Wisconsin and Michigan and persisted only in northern Minnesota (FWS 2007), adjacent to the large

number of wolves in western Ontario. Wolves have since recovered in the western Great Lakes region (WGLR) owing to protection under the Endangered Species Act (ESA) and were delisted in 2007 (FWS 2007). The WGLR wolf population has subsequently undergone several changes in status under the ESA because of legal issues with the delisting process. There is general agreement that the WGLR wolf population has reached sustainable numbers and is thriving, but the genetic identity of these wolves is uncertain, with studies suggesting that they are either hybrids of grey wolves and coyotes (Lehman *et al.* 1991; Roy *et al.* 1994; Leonard & Wayne 2008; Koblmuller *et al.* 2009) or hybrids of grey wolves and eastern wolves (Mech & Federoff 2002; Wheeldon & White 2009; Wilson *et al.* 2009; Fain *et al.* 2010). The fundamental issue underlying this controversy is whether the Old World evolved grey wolf hybridizes with a New World evolved wolf (i.e. *C. lycaon*) or the coyote. This taxonomic uncertainty makes the development and implementation of conservation policies difficult; a consensus based on the careful interpretation and synthesis of data from genetic, morphological and ecological studies is clearly needed (Schwartz & Vucetich 2009).

Here, we genetically characterize sympatric wolves and coyotes from the WGLR using multiple genetic markers to clarify the taxonomic status of *Canis* species in this region. We specifically tested the hypothesis of wolf–coyote hybridization to assess whether there is evidence of current gene flow. We predicted that if WGLR wolves derive from wolf–coyote hybridization, there should be evidence of current gene flow with sympatric coyotes. Alternatively, we predicted that if WGLR wolves derive from grey–eastern wolf hybridization, there should be evidence of mtDNA and Y chromosome haplotypes specific to eastern wolves (i.e. not of grey wolf origin and absent in coyotes), and an absence of gene flow with sympatric coyotes. We discuss our findings in relation to other biological data and comment on conservation and management implications.

Materials and methods

Samples and DNA extraction

We obtained samples from 410 wild canids (i.e. wolves and coyotes) collected between 1998 and 2009 from western Ontario and the western Great Lakes states of Minnesota, Wisconsin, and Michigan (Fig. 1). For this study, these sampling locations collectively represent the western Great Lakes region (WGLR). Many of the samples were previously analysed by Wheeldon (2009). The samples from the western Great Lakes states were

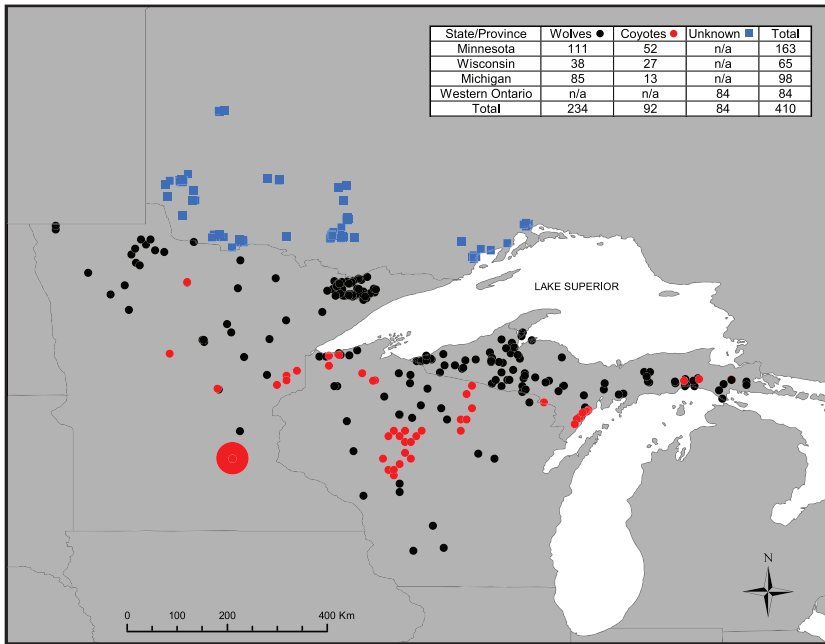


Fig. 1 Geographical distribution of canid samples ($n = 410$) in the western Great Lakes Region. Inset table gives sample frequency by species and province/state. Note that some samples may be overlapping. The oversized dot indicates a large number ($n = 44$) of coyote samples collected from west of Minneapolis, MN.

classified based on phenotype as wolves ($n = 234$) or coyotes ($n = 92$) by the collector. A large number of coyote samples ($n = 44$) were collected from west of Minneapolis, which although located south of primary wolf range in Minnesota, is within dispersal distance of wolves (Merrill & Mech 2000). The samples collected from western Ontario ($n = 84$) were not provided with phenotypic classifications and thus were treated as unknowns (i.e. wolf or coyote). We did not possess an adequate reference set of dog (*Canis familiaris*) samples for inclusion in our analysis; therefore, we excluded two samples of suspected wolf–dog hybrids from Wisconsin (see Discussion).

Samples included whole tissue ($n = 210$), blood on FTA cards ($n = 84$), serum ($n = 62$), hairs ($n = 2$) and previously extracted DNA ($n = 52$). We extracted DNA from samples using a DNeasy Blood and Tissue Kit (Qiagen), monitoring for contamination with multiple negative controls. We determined gender by amplification of the Zfx/Sry primer pairs P1-5EZ/P2-3EZ (Aasen & Medrano 1990) and Y53-3C/Y53-3D (Fain & LeMay 1995) or the Zfx/Zfy primers LGL-331 and LGL-335 (Shaw *et al.* 2003). The serum samples were assumed to be low template and were quantified to concentrations of >250 pg (many >500 pg) per 4 μ L of DNA based on gel imaging using Zfx/Sry PCR products.

Autosomal microsatellite genotyping

We amplified 12 autosomal nuclear microsatellite loci in three multiplex reactions (four loci per multiplex) for each sample (Ostrander *et al.* 1993, 1995: cxx225,

cxx200, cxx123, cxx377, cxx250, cxx204, cxx172, cxx109, cxx253, cxx442, cxx410, cxx147) in a total reaction volume of 15 μ L using 5 ng (or 2–4 μ L) of genomic DNA, 200 μ M dNTPs, 1 \times amplification buffer, 1.5 mM MgCl₂, 0.2–0.3 μ M of forward and reverse primer and 0.05 units of Taq polymerase (BRL). BSA was included in the reaction for some samples. Products were amplified under the following conditions: 94°C for 5 min; 94°C for 30 s, 56–58°C for 1 min, 72°C for 1 min, 30 cycles; 60°C for 45 min. Amplified products were purified through ethanol precipitation and analysed on a MegaBACE 1000 (GE Healthcare) or an ABI3730 (Applied Biosystems). Alleles were scored in Genemarker (v1.7; SoftGenetics LLC), accounting for allele shifts between instruments with multiple control samples. Low amplifying homozygous alleles and uncertain allele scores (e.g. over-saturated, pull-up, off-ladder) were re-amplified to confirm the genotype for the locus.

Mitochondrial DNA control region sequencing

We used primers AB13279 (5'-GAA GCT CTT GCT CCA CCA TC-3'; Pilgrim *et al.* 1998) and AB13280 (5'-GGG CCC GGA GCG AGA AGA GGG AC-3'; Wilson *et al.* 2000), to amplify a 343–347-bp fragment of the control region of the mitochondrial DNA (mtDNA). The control region was amplified in a total reaction volume of 20 μ L per tube using 5 ng (or 2–4 μ L) of genomic DNA, 200 μ M dNTPs, 1 \times amplification buffer, 1.5 mM MgCl₂, 0.2 μ M of each primer and 0.05 units of Taq polymerase (BRL). BSA was included in the reaction for some samples. Products were amplified under the

following conditions: 94°C for 5 min, 60°C for 30 s, 72°C for 30 s; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 30 cycles; 94°C for 30 s, 60°C for 30 s, 72°C for 2 min. For some samples, we amplified the control region using primers ThrL (5'-GAA TTC CCC GGT CTT GTA AAC C-3') and DL-Hcan (5'-CCT GAG GTA AGA ACC AGA TG-3') described in Leonard *et al.* (2002), under similar conditions to those above. PCR products were cleaned using Exosap-IT (USB Corporation) prior to sequencing on a MegaBACE 1000 (GE Healthcare).

Y chromosome microsatellite genotyping

For male samples, we amplified four Y chromosome microsatellite loci using published primers (Sundqvist *et al.* 2001: MS34A, MS34B, MS41A, MS41B) in a total reaction volume of 15 µL using 5 ng (or 2–4 µL) of genomic DNA, 200 µM dNTPs, 1× amplification buffer, 1.5 mM MgCl₂, 0.1–0.21 µM of forward and reverse primer and 0.05 units of Taq polymerase (BRL). BSA was included in the reaction for some samples. Products were amplified under the following conditions: 94°C for 5 min; 94°C for 30 s, 58–60°C for 1 min, 72°C for 1 min, 30 cycles; 60°C for 45 min. Amplified products were purified through ethanol precipitation and analysed on an ABI3730 (Applied Biosystems), and alleles were scored in Genemarker (v1.7; SoftGenetics LLC).

Genetic analysis

We obtained autosomal microsatellite genotypes based on 10 ($n = 2$), 11 ($n = 5$) and 12 ($n = 403$) loci. We analysed the microsatellite genotype data using Structure (v2.3, Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009), which uses a Bayesian approach to infer the number of populations and estimates admixture proportions by assigning each multilocus genotype a probability of membership in each genetic cluster. The admixture model of Structure was run for $K = 1$ to $K = 7$ with five repetitions of 10^6 iterations following a burn-in period of 250 000 iterations for each K . We implemented the I-model of Structure and inferred the parameter alpha. The posterior probability (Ln P[D]) for a given K was computed by averaging the posterior probabilities across the five runs for that K . We determined the number of populations K based on a combination of quantitative criteria (Pritchard *et al.* 2000 : Ln P[D]; Evanno *et al.* 2005: ΔK) and consideration of the overall individual ancestry assignments and biological significance. The data were run through Structure again for the selected value of K with 10 repetitions, and the individual admixture proportions (Q -values) were taken from the run having the highest posterior probability and lowest variance. We collected information on the

90% probability intervals of individual assignments (ANCESTDIST = 1) to assess their credibility. We classified individuals with $Q \geq 0.8$ as belonging to one cluster and those with $Q < 0.8$ as admixed (e.g. Vaha & Primmer 2006).

To supplement the results from Structure, we performed a nonmodel-based factorial correspondence analysis (FCA) on the microsatellite data for individual canids using GENETIX (v4.05; Belkhir *et al.* 2004). Two factorial components FC-1 and FC-2, which accounted for 7.94% and 3.07% of the total inertia, respectively, were plotted to visualize the relative clustering of animals.

A 222–229- bp (note C98 = 200 bp) highly informative region of the mtDNA control region was sequenced. We edited mtDNA sequences in Bioedit (Hall 1999) and assigned haplotypes, denoted as C(n), some of which correspond to sequences previously described by Wilson *et al.* (2000). A previously identified diagnostic insertion/deletion was used to distinguish between mtDNA sequences evolved in grey wolves and those evolved in coyotes and eastern wolves (Pilgrim *et al.* 1998; Wilson *et al.* 2000). Mitochondrial DNA sequences not previously described have been deposited in Genbank (see Results section).

We combined the genotypes of the four Y chromosome microsatellite loci into haplotypes because they are inherited as a single unit (Sundqvist *et al.* 2001). We classified haplotypes based on the allele present at the diagnostic locus MS41A, for which allele 208 is specific to grey wolves and dogs, and alleles 212–216 are specific to coyotes and eastern wolves (Sundqvist *et al.* 2001, 2006; Shami 2002; Hailer & Leonard 2008; Fain *et al.* 2010).

For consistency and ease of comparison in the results and discussion sections, we hereafter refer to mtDNA and Y chromosome haplotypes as being evolved in either grey wolves (GW) or coyotes and eastern wolves (C/EW).

Results

Microsatellite genotypes

The I-model of Structure inferred two genetic clusters based on quantitative criteria (Fig. 2a); higher values of K produced splitting within wolf and coyote clusters that was not easily interpreted biologically and apparent geographical partitioning showed overlap. The two genetic clusters identified by Structure corresponded to wolves and coyotes and had minimal amounts of admixture (Fig. 2b). The results of the FCA were concordant with the results from Structure, revealing two distinct clusters separated along the

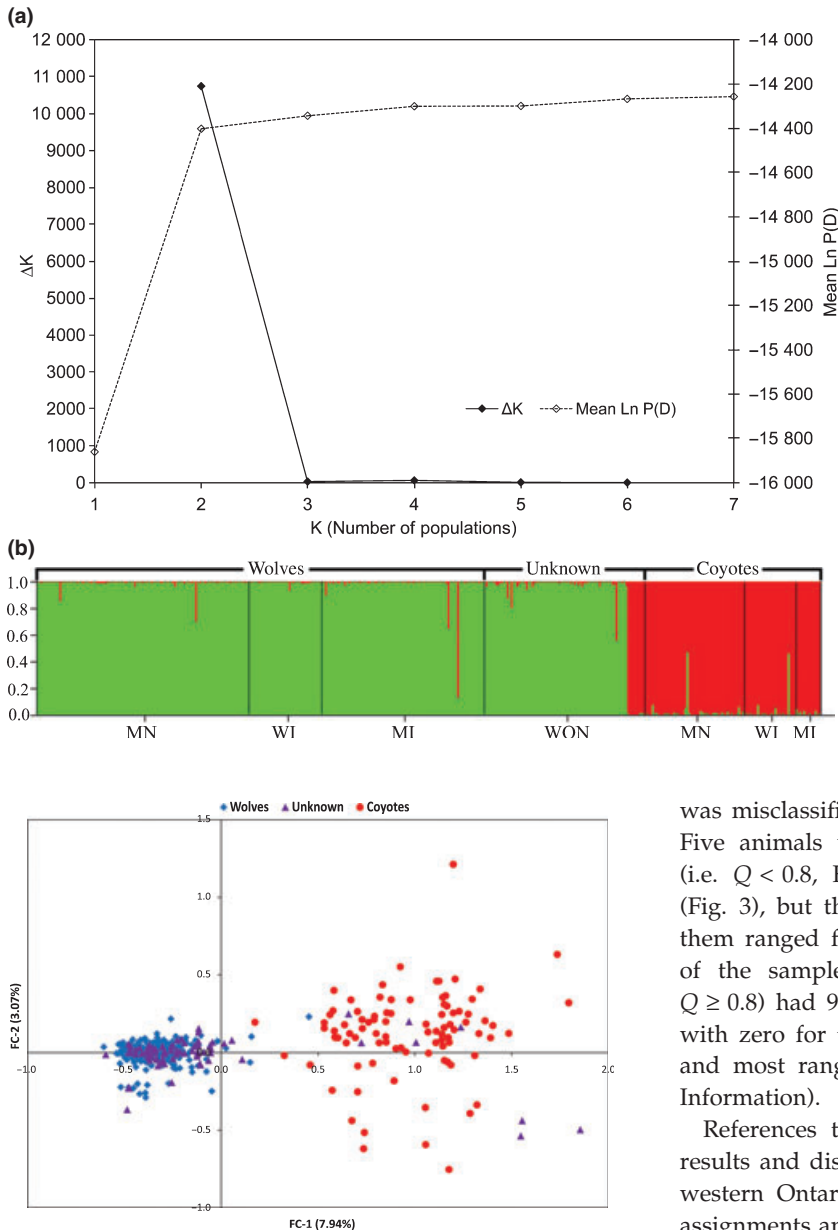


Fig. 2 Structure analysis of autosomal microsatellite genotype data: (a) plot of K determination criteria values, ΔK and Ln P(D) ; (b) plot of individual proportional memberships to the populations inferred at $K = 2$ (WON, western Ontario; MN, Minnesota; WI, Wisconsin; MI, Michigan).

Fig. 3 Factorial correspondence analysis (FCA) of autosomal microsatellite genotype data. Note that the FCA plot is zoomed in to clearly visualize individual sample clustering patterns, resulting in two coyote samples being out of view that diverged along the vertical axis from the coyote cluster.

horizontal axis, represented by wolves and coyotes (Fig. 3). The majority of samples from western Ontario were assigned as wolves by Structure, but some were assigned as coyotes and one as admixed (Fig. 2b), which is evident in the FCA plot (Fig. 3). One animal from Michigan that was phenotypically classified as a wolf was assigned as a coyote in Structure (Fig. 2b), which was also evident in the FCA plot (Fig. 3), therefore we assume that this individual

was misclassified based on phenotype (see Discussion). Five animals were assigned as admixed in Structure (i.e. $Q < 0.8$, Fig. 2b), which is reflected in the FCA (Fig. 3), but the 90% probability intervals for three of them ranged from zero to one for both clusters. None of the samples assigned as wolves or coyotes (i.e. $Q \geq 0.8$) had 90% probability intervals that overlapped with zero for the cluster to which they were assigned, and most ranged from >0.8 to 1 (refer to Supporting Information).

References to samples as wolves or coyotes in the results and discussion sections, including samples from western Ontario, are hereafter based on the Structure assignments and not phenotypic classification.

Mitochondrial DNA haplotypes

We observed 33 mtDNA haplotypes in 410 canid samples (males, $n = 214$; females, $n = 196$); five were of GW origin ($n = 105$) and 28 were of C/EW origin ($n = 305$) (Table 1). We observed GW and C/EW haplotypes in wolves and only C/EW haplotypes in coyotes, but the haplotypes observed in wolves and coyotes were mutually exclusive (Table 1). We observed GW and C/EW haplotypes in the admixed individuals; two had C/EW haplotypes that were observed in wolves (C3 and C13), one had a C/EW haplotype observed in coyotes (C48), one had a unique C/EW haplotype (C113) and one had a unique GW haplotype (C96) (Table 1). We observed

mtDNA haplotype	Frequency	Females	Males	STRUCTURE assignment	Genbank accession
C1	1	1	n/a	Coyote (1)	AY267718
C3	64	29	35	Wolf (63), Admixed (1)	AY267720
C5	8	3	5	Coyote (8)	AY267722
C9	12	5	7	Coyote (12)	AY267726
C13	139	64	75	Wolf (138), Admixed (1)	AY267730
C14	4	1	3	Coyote (4)†‡	AY267731
C19	10	4	6	Coyote (10)	AY267736
C22+	91	44	47	Wolf (91)	FJ687608
C23+	7	3	4	Wolf (7)	FJ687609
C41	13	5	8	Coyote (13)	FJ889990
C43	1	1	n/a	Coyote (1)	FJ889992
C48	5	4	1	Coyote (4), Admixed (1)	FJ687613
C70	1	1	n/a	Coyote (1)	FJ687614
C72	4	1	3	Coyote (4)	FJ687615
C73	1	1	n/a	Coyote (1)	GU014247
C75	6	4	2	Coyote (6)	GU014248
C95+	1	1	n/a	Wolf (1)	FJ687610
C96+	1	n/a	1	Admixed (1)	FJ687611
C97+	5	1	4	Wolf (5)	FJ687612
C98	5	3	2	Coyote (5)	FJ687616
C99	4	3	1	Coyote (4)	FJ687617
C107	7	4	3	Coyote (7)	GU014249
C108	2	1	1	Coyote (2)	GU014250
C109	1	1	n/a	Coyote (1)	GU014251
C110	6	4	2	Coyote (6)	GU014252
C111	2	2	n/a	Coyote (2)	GU014253
C112	2	1	1	Coyote (2)	GU014254
C113	1	n/a	1	Admixed (1)	GU014255
C114	2	1	1	Coyote (2)	GU014256
C115	1	1	n/a	Coyote (1)	GU014257
C116	1	1	n/a	Coyote (1)	GU014258
C117	1	1	n/a	Coyote (1)	GU014259
C118	1	n/a	1	Coyote (1)	GU014260
Total	410	196	214		

Table 1 Canid mitochondrial DNA haplotype frequencies and Genbank accession numbers

†Sequences of grey wolf origin; all other sequences are of coyote or eastern wolf origin. The STRUCTURE cluster to which samples having the haplotype of interest were assigned is indicated.

‡The sample that was phenotypically classified as a wolf and genetically assigned as a coyote.

two C/EW haplotypes in wolves in high frequency, but neither was observed in coyotes (C3 and C13, Table 1). The sample phenotypically classified as a wolf that was genetically assigned as a coyote had a C/EW haplotype (C14) that was not observed in wolves (Table 1).

Y chromosome haplotypes

We obtained Y chromosome microsatellite genotypes at 4 loci for 97.7% of males ($n = 209$) and identified 29 haplotypes; 11 were of GW origin (i.e. allele 208 at locus MS41A) and 18 were of C/EW origin (i.e. alleles 212–216 at locus MS41A) (Table 2). We observed GW and C/EW haplotypes in both wolves and coyotes,

but only two were shared between populations; one GW haplotype (FG) and one C/EW haplotype (AA) (Table 2). In the admixed individuals, we observed GW and C/EW haplotypes; two had C/EW haplotypes that were observed in coyotes (AJ and CK), and one had a GW haplotype observed in wolves (CE) (Table 2). We observed 10 GW haplotypes in wolves, but only 2 C/EW haplotypes were observed in wolves (BB and AA); BB was observed in high frequency and exclusively in wolves (Table 2). We observed 17 C/EW haplotypes in coyotes, but only 2 GW haplotypes were observed in coyotes (FG and FL); FL was observed in one coyote but no wolves (Table 2).

Y chromosome haplotype	34a	34b	41a	41b	Frequency	STRUCTURE assignment
AA	172	180	212	212	6	Coyote (1), Wolf (5)
AF	172	180	208	222	11	Wolf (11)
AG	172	180	208	224	2	Wolf (2)
AI	172	180	214	214	1	Coyote (1)
AJ	172	180	212	214	6	Coyote (5), Admixed (1)
AQ	172	180	212	218	1	Coyote (1)
AR	172	180	212	216	3	Coyote (3)
BB	170	182	212	226	82	Wolf (82)
BP	170	182	212	222	3	Coyote (3)
CC	172	178	208	214	9	Wolf (9)
CD	172	178	214	210	3	Coyote (3)
CE	172	178	208	216	23	Wolf (22), Admixed (1)
CF	172	178	208	222	2	Wolf (2)
CI	172	178	214	214	5	Coyote (5)
CK	172	178	214	216	4	Coyote (3), Admixed (1)
CM	172	178	214	218	7	Coyote (7)
CO	172	178	212	220	1	Coyote (1)
CR	172	178	212	216	1	Coyote (1)
CS	172	178	208	226	20	Wolf (20)
CT	172	178	208	220	4	Wolf (4)
CU	172	178	214	212	3	Coyote (3)
CV	172	178	214	222	2	Coyote (2)
CX	172	178	216	220	1	Coyote (1)
DC	172	176	208	214	1	Wolf (1)
DQ	172	176	212	218	1	Coyote (1)
FG	174	178	208	224	4	Coyote (2), Wolf (2)
FL	174	178	208	218	1	Coyote (1)
FT	174	178	208	220	1	Wolf (1)
KA	172	182	212	212	1	Coyote (1)

Table 2 Canid Y chromosome haplotypes based on microsatellite genotypes at 4 loci

First letter of haplotype indicates allele combination for MS34A and MS34B, and second letter of haplotype indicates allele combination for MS41A and MS41B. We classified haplotypes based on the allele present at locus MS41A: allele 208 = grey wolf origin; alleles 212–216 = coyote or eastern wolf origin. Frequency of observation for each haplotype is indicated. The STRUCTURE cluster to which samples having the haplotype of interest were assigned is indicated.

Synthesis of genetic data

We observed male individuals with a GW mtDNA and Y chromosome haplotype combination or a C/EW mtDNA and Y chromosome haplotype combination (Table 3), with the former observed exclusively in wolves and the latter observed in both wolves and coyotes (refer to Supporting Information). The combination of a GW mtDNA and a C/EW Y chromosome haplotype was observed (Table 3) exclusively in wolves, and the combination of a C/EW mtDNA and a GW Y chromosome haplotype was observed (Table 3) predominantly in wolves, but also in three coyotes (refer to Supporting Information).

We obtained Y chromosome haplotypes for three of the four males identified as admixed by Structure, revealing two males with a C/EW mtDNA and Y chro-

some haplotype combination (C48 with AJ; C113 with CK), and one male with a C/EW mtDNA and GW Y chromosome haplotype combination (C13 with CE) (refer to Supporting Information). Thus, only 1 admixed individual identified based on autosomal data had mixed ancestry based on mtDNA and Y chromosome data, but notably the C/EW mtDNA haplotype it had (C13) was not observed in coyotes. Conversely, we note that the individuals with mixed ancestry based on mtDNA and Y chromosome data did not exhibit admixture based on autosomal data.

Discussion

We genetically characterized *Canis* from the WGLR using maternal, paternal, and bi-parental genetic markers and demonstrated a clear genetic distinction

Table 3 Mitochondrial DNA and Y chromosome haplotype composition of male canids

mtDNA haplotype	Y chromosome haplotypes
C3	AA (2), AF (2), AG (2), BB (21), CC (2), CE (1), CS (2), CT (2)
C5	CK (1), CM (3), CV (1)
C9	AR (2), CI (1), CU (2), FL (1), KA (1)
C13	AA (1), AF (5), BB (43), CC (1), CE (14), CF (2), CS (6), CT (1), DC (1), FT (1)
C14	CM (1), CV (1)
C19	AJ (2), AQ (1), CD (1), CI (1), FG (1)
C22	AA (2), AF (2), BB (15), CC (6), CE (6), CS (11), CT (1), FG (2)
C23	AF (1), BB (1), CE (2)
C41	BP (1), CD (1), CM (3), CX (1), DQ (1), FG (1)
C48	AJ (1)
C72	AI (1), AJ (1), BP (1)
C75	CR (1), CU (1)
C97	AF (1), BB (2), CS (1)
C98	CI (2)
C99	AA (1)
C107	BP (1), CD (1), CO (1)
C108	AR (1)
C110	AJ (2)
C112	CK (1)
C113	CK (1)
C114	CK (1)
C118	CI (1)

Haplotypes of grey wolf origin are in bold font; haplotypes of coyote or eastern wolf origin are in normal font. Frequency indicated in parentheses.

between sympatric wolves and coyotes in the region. We observed C/EW haplotypes in wolves that were not observed in coyotes (i.e. C3, C13, BB). This trend has been noted previously (e.g. Lehman *et al.* 1991; Fain *et al.* 2010) and requires careful interpretation. There are two competing hypotheses that could explain the observation of C/EW haplotypes in wolves but not in coyotes in the WGLR: (i) historic introgression from coyotes into grey wolves and the loss of those haplotypes from the coyote population (e.g. Lehman *et al.* 1991; Koblmüller *et al.* 2009) or (ii) those haplotypes derive from historic and/or ongoing hybridization with a New World evolved wolf species, synonymously referred to as the eastern wolf or Great Lakes wolf (e.g. Wilson *et al.* 2000; Leonard & Wayne 2008; Wheelton & White 2009; Fain *et al.* 2010). The loss of several haplotypes from an expanding coyote population seems unlikely, and the C/EW haplotypes observed in WGLR wolves (i.e. C3, C13, BB) have not been observed in western coyote populations (Hailer & Leonard 2008; Koblmüller *et al.* 2009), or in coyotes sampled east of the region of wolf-coyote hybridization in southeastern

Ontario (Kays *et al.* 2010; Rutledge *et al.* 2010; Way *et al.* 2010; Wheelton *et al.* 2010). Phylogenetic analysis reveals that C3, and also C1, are divergent from coyote sequences and thus plausibly of eastern wolf origin (Wilson *et al.* 2000, 2003; Leonard & Wayne 2008 see Wheelton & White 2009; Fain *et al.* 2010). Koblmüller *et al.* (2009) reported that the 'Great Lakes wolf' mtDNA sequences were within the range of diversity of the coyote-clade sequences, but their observation of coyote-clade sequences in wolves and not coyotes is congruent with those sequences deriving from a New World evolved wolf species, the eastern wolf, that shares a close evolutionary relationship with the western coyote (Wilson *et al.* 2000; Fain *et al.* 2010). The presence of C13 in wolves and not in coyotes supports the suggestion of Wheelton & White (2009), and Fain *et al.* (2010) that this 'coyote-clade' sequence is of eastern wolf origin; probably, the result of incomplete lineage sorting, or the introgression of an ancestral coyote haplotype and subsequent divergence to become eastern wolf specific. We suggest that the C/EW Y chromosome haplotypes observed in wolves and not in coyotes (Koblmüller *et al.* 2009; Fain *et al.* 2010; BB from this study) represent eastern wolf haplotypes. Although we cannot rule out the occurrence of historic hybridization between WGLR wolves and coyotes, given the preceding discussion and the lack of contemporary interbreeding that we documented based on autosomal data, we suggest that the C/EW haplotypes observed in WGLR wolves are derived from the New World evolved eastern wolf, and not from coyotes. We suggest that haplotypes C1, C3, C13, AA and BB are of eastern wolf origin given their presence in wolves and absence in western (i.e. nonhybridizing) coyote populations. The presence of mtDNA haplotype C1 and Y chromosome haplotype AA in eastern coyotes (e.g. Rutledge *et al.* 2010) is consistent with them being of eastern wolf origin, given that eastern coyotes have derived from eastern wolf-coyote hybridization (Schmitz & Kolenosky 1985; Wilson *et al.* 2009; Kays *et al.* 2010; Wheelton *et al.* 2010). Given our interpretation of the data, we observed the following: (i) mixed ancestry in WGLR wolves deriving from hybridization between grey wolves and eastern wolves; and (ii) no evidence of coyote introgression in wolves based on mtDNA or Y chromosome data, but coyotes showed some evidence of low-level introgression from wolves (and possibly dogs) based on these markers (i.e. C1, AA, FG, FL).

We observed haplotype C1 in one coyote from Michigan, representing our only case of wolf mtDNA introgression in coyotes, and although C1 was not observed in wolves in this study, it has been observed in historic and contemporary WGLR wolves (Leonard & Wayne 2008; Wheelton & White 2009; Fain *et al.*

2010) and is present in contemporary eastern wolves and eastern coyotes (e.g. Rutledge *et al.* 2010; Way *et al.* 2010). This particular coyote may have descended from wolf–coyote hybridization that occurred in situ, or more likely to the east in southeastern Ontario. A similar interpretation applies to the coyote observed with Y chromosome haplotype AA, which was present in wolves in this study, and is also found in eastern wolves and eastern coyotes (Rutledge *et al.* 2010).

We note that three of the GW Y chromosome haplotypes that we observed (FG, FL and FT) have been commonly observed in dogs (h71, h39 and h31, respectively in Sundqvist *et al.* 2006; allele data obtained from J. Leonard). It is unknown if the presence of haplotypes FG and FT in wolves (FT in Mexican grey wolves, Hailer & Leonard 2008; this study) and dogs is because of shared ancestry or hybridization, but in the absence of more data, we assume that these two haplotypes represent wolf ancestry in our study samples. Conversely, the absence of haplotype FL in wolves but presence in a coyote in our study may be indicative of coyote–dog hybridization, but this is confused by the observation of FL in a captive red wolf (Hailer & Leonard 2008). The lack of an adequate reference set of dog microsatellite genotypes limited our ability to assess hybridization between wild canids and dogs, but we observed no dog mtDNA haplotypes and found minimal evidence of potential dog introgression based on Y chromosome data. Furthermore, neither of the two suspected wolf–dog hybrids (both female) that we excluded from our analysis possessed dog mtDNA haplotypes, although Fain *et al.* (2010) reported some evidence of wolf–dog hybridization based on autosomal data. Fain *et al.* (2010) found no evidence of coyote–dog hybridization based on autosomal data, but did observe putative GW or dog Y chromosome haplotypes (i.e. allele 208 at locus MS41A) in coyotes that were not observed in wolves. The data suggest that if wolf–dog or coyote–dog hybridization occurs in the WGLR, it is likely asymmetric (i.e. male dog breeding with female wolf or coyote), and the offspring rarely backcross to the wild canid populations. As discussed by Hilton (1978), the phase shift in the breeding cycle of coyote–dog hybrids that results in pups being born mid-winter, the lack of parental care provision typical of male dogs, and the loss of adaptive characteristics of wild *Canis*, may be sufficient to prevent the backcrossing of coyote–dog hybrids with wild *Canis* populations because of reduced survival and competitive disadvantage. Further investigation of potential dog introgression into wolf or coyote populations in the WGLR and elsewhere is warranted.

Our findings contrast with previous interpretations that suggested wolf–coyote hybridization was prevalent in the WGLR, with introgression from coyotes into

wolves (e.g. Lehman *et al.* 1991; Koblmuller *et al.* 2009); however, this apparent conflict results from the failure of previous studies to distinguish between eastern wolf and coyote mtDNA haplotypes. We suggest that the apparent ‘intensive recent and ongoing gene flow’ that Koblmuller *et al.* (2009) observed between ‘Great Lakes wolves’ and coyotes is probably geographically restricted primarily to areas east of the WGLR, such as in southeastern Ontario, where wolf–coyote hybridization has been documented (e.g. Schmitz & Kolenosky 1985; Wilson *et al.* 2009), and there is putative evidence of eastern wolves bridging gene flow between coyotes and grey wolves (Rutledge *et al.* 2010). This is supported by the observation that the mtDNA haplotype sharing between ‘Great Lakes wolves’ and coyotes observed by Koblmuller *et al.* (2009) does not include the two predominant C/EW haplotypes that they observed in WGLR wolves (GL2 and GL10) that were also the only two C/EW haplotypes observed in WGLR wolves in this study (i.e. referred to here as ‘C3’ and ‘C13’, see Wheelton & White 2009), which are interpreted as eastern wolf haplotypes (Fain *et al.* 2010; this study). Rather the observed mtDNA sharing was primarily of haplotypes common in wolves and coyotes found to the east of the WGLR (e.g. GL1, GL11, GL13 and GL16 in Koblmuller *et al.* 2009 correspond to C1, C19, C14 and C9, respectively in Rutledge *et al.* 2010; see Wheelton & White 2009). We also note the minimal amount of Y chromosome haplotype sharing among ‘Great Lakes wolves’ and coyotes ($n = 3$) observed by Koblmuller *et al.* (2009). The composite genetic data are consistent with WGLR wolves having mixed ancestry deriving from grey wolves and eastern wolves and not coyotes.

Based on our interpretation of the haplotype data, we performed a post hoc contingency table analysis of the mtDNA and Y chromosome haplotype combinations observed in male WGLR canids assigned as wolves in STRUCTURE ($Q \geq 0.8$). Similar to Fain *et al.* (2010), we observed evidence of asymmetric breeding with respect to grey wolf and eastern wolf haplotypes, with combinations of mtDNA and Y chromosome haplotypes of the same ancestry being observed more than expected (Table 4). Although the mtDNA and Y chromosome data appear to indicate that nonhybridized grey wolves and eastern wolves exist in the WGLR (Table 3), our autosomal microsatellite data indicates that they cluster in the same population (Fig. 2b). Furthermore, wolf population substructure that we observed at $K = 3$ did not have any apparent relationship to haplotype composition (data not shown), suggesting that the WGLR wolf population essentially represents a single interbreeding population (e.g. Fain *et al.* 2010), presumably having undergone extensive backcrossing to both parental species over time.

Table 4 Contingency table analysis of mitochondrial DNA and Y chromosome haplotypes observed in males assigned as wolves in STRUCTURE ($Q \geq 0.8$): expected frequencies in brackets; χ^2 statistic = 9.71251, d.f. = 1, $P < 0.005$

Ancestry/marker type	Grey wolf mtDNA	Eastern wolf mtDNA	Total
Grey wolf Y chromosome	33 (23.8)	38 (47.2)	71
Eastern wolf Y chromosome	20 (29.2)	67 (57.8)	87
Total	53	105	158

The 90% probability intervals of three of the five individuals assigned as admixed by Structure ranged from zero to one for both clusters, suggesting that the assignments of some putatively admixed individuals are likely not representative of true hybridization between wolves and coyotes, which is supported by the lack of admixture based on haplotype data for these individuals. The reliability of some individual assignments may be affected by the absence of certain populations in the analysis, such as grey wolves to the north and west, and eastern coyotes to the east, both of which likely have some gene flow with WGLR *Canis* populations (Koblmuller *et al.* 2009; Wheeldon 2009; Fain *et al.* 2010). Regardless of this potential effect on specific individual assignments, our results show a clear genetic separation and minimal amounts of admixture between wolf and coyote populations that is corroborated by other studies of WGLR *Canis* populations that incorporated other potential contributing *Canis* populations (Koblmuller *et al.* 2009; Wheeldon 2009; Fain *et al.* 2010). We will further investigate wolf and coyote population substructuring with increased data sets.

Aggressive interactions between wolves and coyotes have been reported in Wisconsin, including the killing of coyotes by wolves (Thiel 2006), but reports also exist of genial behavioural interactions between wolves and coyotes in Wisconsin (Thiel 2006), presenting the possibility for sexual interaction between them. Maternal and paternal genetic markers indicate the low-level introgression of wolf (or dog; Y chromosome only) genes in coyotes in the WGLR, but this may reflect past hybridization and is not consistent with ongoing gene flow, which appears minimal (Fig. 2b). We note that the discordance we observed in admixture detected from autosomal data vs. mtDNA and Y chromosome data was also observed by Fain *et al.* (2010).

The finding based on genetic analysis that hybridization between sympatric WGLR wolves and coyotes is uncommon is supported by morphological analyses of animals from this region. There is no convincing evidence to support wolf-coyote hybridization in the WGLR based on morphology (but see Lawrence &

Bossert 1969), ecology or behaviour, and the wolves of Minnesota and western Ontario are fully wolf-like in those respects (Nowak 2003). Mech & Paul (2008) analysed body mass data from a large sample of Minnesota wolves and reported no evidence of wolf-coyote hybridization, but suggested that the east-west body mass cline they observed supports the hypothesis of hybridization between grey and eastern wolves, as does data from skull measurements (L. D. Mech & R. M. Nowak, unpublished). A broad comparison of coyote body mass across North America revealed that Minnesota coyote body masses were similar to those of western populations, and not of those in the east (Way 2007). Schmitz & Kolenosky (1985) demonstrated using body weight, foot length and skull measurements that Minnesota coyotes were not similar to captive coyote-wolf hybrids, nor were they similar to the southeastern Ontario coyotes that approximated captive coyote-wolf hybrids. Morphological evidence supports wolf-coyote hybridization in southeastern Ontario (Schmitz & Kolenosky 1985; Sears *et al.* 2003) and the New England states (Lawrence & Bossert 1969; Kays *et al.* 2010), but generally not west of these regions. The presumed phenotypic misclassification of a genetically assigned coyote as a wolf in this study may be indicative of the presence of larger eastern coyote-like animals in the WGLR, but determining whether animals like this originate in situ or result from westward dispersal from Ontario requires further investigation. The classification of *Canis* species in the Great Lakes region based on morphology is congruent with the classification based on genetics from this study.

The current WGLR wolf population is composed of grey-eastern wolf hybrids that probably resulted from historic hybridization between the parental species (Wheeldon & White 2009). This hybridization may have occurred during interglacial periods of the Pleistocene when these different wolf species came into contact after prior isolation. Alternatively, this hybridization may have occurred after European colonization, when land clearing and the resultant changes in prey distribution may have broke down postglacial reproductive barriers (Kolenosky & Standfield 1975; Kyle *et al.* 2006). The occurrence of grey-eastern wolf hybridization over a century ago seems plausible given that (i) grey wolves decreased in abundance in the western Great Lakes states because of human persecution since the mid 1800s (FWS 2007) and (ii) eastern wolves likely expanded their range into this region following the northward expansion of white-tailed deer (*Odocoileus virginianus*) (Kolenosky & Standfield 1975; Nowak 2003). These two points suggest that the two species probably came into contact in disproportionate abundances, which could have contributed to hybridization

(e.g. Grant & Grant 1997; Reyer 2008). We note that Koblmüller *et al.* (2009) reported that four historic 'Great Lakes wolves' had wolf Y chromosomes, and personal communication with the corresponding author (J. Leonard, Uppsala University, Sweden) confirmed that these samples were from Michigan and that all four had the 208 allele specific to grey wolves present at locus MS41A. Furthermore, the four historic Michigan wolf samples all had an eastern wolf mtDNA haplotype (GL2/C3: Leonard & Wayne 2008 see Wheeldon & White 2009). This supports the suggestion by Wheeldon & White (2009) that the pre-recovery WGLR wolf population was admixed, deriving from apparent asymmetric hybridization between grey and eastern wolves, but this trend is less apparent in the recovered population presumably because of extensive backcrossing over time.

The genetic data are consistent with Wisconsin and Michigan being re-colonized by wolves from Minnesota (Fain *et al.* 2010; this study), which would have been receiving immigrants from western Ontario and south-eastern Manitoba (Mech 2010). For both wolf and coyote populations in the WGLR, we cannot rule out gene flow from the east in Ontario, with animals possibly crossing near Sault Ste. Marie into the upper Michigan peninsula, given the observation of haplotypes in WGLR canids that are also common in eastern Ontario canids (e.g. Rutledge *et al.* 2010). Thus, we cannot rule out the possibility of eastern coyote-like canids occurring in the WGLR, especially given the observation of an animal from Michigan that was genetically assigned as a coyote but phenotypically classified as a wolf. Regardless, it is clear that sympatric wolves and coyotes in the WGLR are reproductively isolated. The population structure of WGLR wolves suggests high levels of intrapopulation gene flow and connectivity (Koblmüller *et al.* 2009; Wheeldon 2009; Fain *et al.* 2010), and accordingly conservation and management strategies should focus on conserving this genetic connectivity. The combination of grey wolf and eastern wolf genetic material in WGLR wolves may provide adaptive evolutionary potential to changing environmental factors such as climate and habitat, which could impact the abundance and distribution of prey. Although interbreeding between sympatric wolves and coyotes in the WGLR is presently negligible, it is uncertain how environmental changes in the future may affect the relationship between these canids in this region. However, given that WGLR wolves occurred at low densities and sympatric with coyotes for decades prior to their recovery, yet we found no evidence of coyote mtDNA or Y chromosome introgression in wolves, the potential for wolf-coyote hybridization does not seem significant, contrary to the conclusion by Koblmüller *et al.* (2009).

The following conservation and management implications of our findings are important and require attention: (i) grey wolf numbers are probably overestimated in the 48 contiguous United States given that the wolves occupying the WGLR are not pure grey wolves but rather grey-eastern wolf hybrids; and (ii) policies for hybrids are required under the ESA, especially considering that the recovered WGLR wolf population is hybridized but apparently thriving. These points notwithstanding, the WGLR wolf population is managed federally in the United States as a distinct population segment, not as a taxonomic entity, thus its hybrid nature should not affect the estimation of its numbers, nor the total number of wolves in the United States. Genetic data indicate that the pre-recovery WGLR wolf population was already hybridized, but further analysis of historic samples could provide insight into whether this hybridization occurred pre- vs. post-European colonization (i.e. natural vs. human-caused). Although, the conservation merit of a natural hybrid population may be perceived as greater than that of a hybrid population resulting from human influences (Allendorf *et al.* 2001), the cause of hybridization should not preclude sustaining a hybrid wolf population that fills the ecological role of a top canid predator in the WGLR. Similar logic applies to the conservation merit of eastern coyotes (Kays *et al.* 2010; Way *et al.* 2010).

We suggest that wolf recovery efforts have been successful in restoring a thriving wolf population to the WGLR. Accordingly, state management plans should focus on maintaining adequate numbers of wolves to ensure their long-term persistence in the region. The appropriate taxonomic designation for the WGLR hybrid wolves, commonly referred to as 'Great Lakes wolves', is *Canis lupus × lycaon*, replacing previous grey wolf subspecies designations (see Nowak 2009).

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 WGLR samples.

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