

Phylogeography of wolves (*Canis lupus*) in the Pacific Northwest

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Glacial cycles in the late Pleistocene played a dominant role in sculpting the evolutionary histories of many high-latitude organisms. The refugial hypothesis argues that populations retracted during glacial maxima and were isolated in separate refugia. One prediction of this hypothesis is that populations inhabiting different refugia diverged and then, during interglacial periods, rapidly expanded into deglaciated regions. The range of the gray wolf (*Canis lupus*) was modified by these expansion and contraction cycles in the late Pleistocene. Our analyses of variation of mitochondrial control region sequences corroborate previous microsatellite analyses supporting independent evolutionary histories for Coastal and Continental wolves in North America. Coastal wolves represent the remnants of a formerly widespread and diverse southern clade that expanded into coastal Southeast Alaska, likely in the early Holocene. In contrast, extant northern Continental populations appear to be admixed, composed of lineages independently arising from ancestors that persisted in either southern or northern (Beringia) refugia. This pattern of diversification suggests the possibility of 3 temporally independent colonizations of North America by wolves from Asia. Coastal wolves are the last vestige of a formerly widespread phylogroup that largely was extirpated in North America by humans during the last century. The independent phylogeographic history of these Coastal wolves has yet to be characterized. Their distinctiveness among North American wolf populations may warrant a reevaluation of their conservation status and management. DOI: 10.1644/09-MAMM-A-036.1.

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Pleistocene biogeographic events were likely a major influence in generating modern species diversity and determining community composition, especially at higher latitudes (Lovette 2005; Weir and Schluter 2007). The Pleistocene epoch was a time of dramatic oscillations in climate with an unprecedented cycle of global cooling about every 100,000 years that generated continental glaciations in boreal regions (Berger 1984). Climatic warming, with conditions more akin to those of the Holocene, periodically interrupted the cold glacial advances and profoundly influenced the evolutionary histories of organisms in the northern latitudes (Hewitt 1996, 2001; Webb and Bartlein 1992). In North America the mammals of coastal Southeast Alaska were influenced heavily by a complex history of persistence and divergence in Pleistocene refugia, post-Pleistocene recolonization and contact among divergent lineages, and recently, contemporary fragmentation of habitat (Cook et al. 2001).

Full glacial advances separated North America into at least 2 major unglaciated regions. Well recognized are the lower

latitude areas south of the Laurentide Ice Sheet. Perhaps less well known is the high-latitude Beringian subcontinent that joined North America and Asia but was disjunct from lower latitudes south of the reaches of glacial ice (Hopkins 1967). Investigators have long argued for additional ice-free regions at high latitudes (Fedorov and Stenseth 2002; Rand 1954), along the North Pacific coast (Byun et al. 1997, 1999; Heaton et al. 1996; Moodie and Reimchen 1976; O'Reilly et al. 1993), along the east coast, or in other southern regions (Rand 1954). Debate and uncertainty surround estimates of the number and location of these potential refugia and their significance in shaping extant diversity throughout the higher latitudes of North America.

The fossil record indicates that the gray wolf (*Canis lupus*) first arrived in North America from Eurasia approximately 500,000 years ago, likely crossing the Bering Land Bridge



when the region was ice free during a full glacial event (Nowak 1979). Since then at least 3 major glacial advances exposed the Bering Land Bridge and provided a corridor conducive to the repeated exchange of Asian and North American wolf populations (Waltari et al. 2007). The question remains as to how climate change (Gates 1993) and related admixture of Asian and North American wolves might have shaped geographic variation in North American wolves. During the last glaciation (the Wisconsin, beginning approximately 100,000 years before present), the gray wolf occurred in both Beringia (Guthrie 1968; Nowak 1979) and in a southern refugium or series of refugia (Klein 1965; Nelson and Madsen 1986; Nowak and Paradiso 1983). Studies of morphology uncovered geographical variation consistent with the isolation of wolf populations into separate refugia (Nowak 1995), but previous genetic analyses of wolves in North America using data from the mitochondrial DNA (mtDNA) control region uncovered little geographic structure (Vilà et al. 1999; Wayne et al. 1992, 1995). Roy et al. (1994) proposed that separation of wolf populations by glacial ice sheets led to limited differentiation that was obscured by subsequent gene flow during interglacial periods. Vilà et al. (1999) suggest that the repeated expansion and contraction of wolf populations to refugia throughout the Pleistocene, together with changes in distribution of suitable habitat, contributed to the overall lack of observed phylogeographic structure.

Earlier studies of mitochondrial variation, however, did not focus on North Pacific Coastal wolves, a subset of wolves that is genetically distinct from nearby continental populations based on more variable nuclear microsatellite loci (Carmichael et al. 2007; Weckworth et al. 2005). This distinctive signal likely reflected geographic isolation of Coastal wolf populations and was consistent with the recurring signature of a North Pacific coastal–continental phylogeographic split detected across a number of other North American taxa (Cook et al. 2001; Peacock et al. 2007).

Following the late Wisconsin glacial period, some mammals repopulated Southeast Alaska from regions south of the glacial extent, because the glaciated Coast Range to the north and northeast apparently presented a physical barrier to access from the interior of Alaska and Canada (Cook et al. 2006; Pedersen 1982). Wolves might have followed deer northward into coastal southeastern Alaska from a southern refugium (Klein 1965; but see Latch et al. 2008). In contrast, wolves originating from the northern Beringian refugium (Guthrie 1968; Nowak 1979; Pedersen 1982) occupied the remainder of Alaska. The North Pacific coast is a region of contact between mammalian postglacial colonizers that originated from independent Pleistocene refugia (MacDonald and Cook 2007). Lessa et al. (2003) uncovered “genetic footprints” of expansion for dusky shrews (*Sorex monticolus*), long-tailed voles (*Microtus longicaudus*), flying squirrels (*Glaucomys sabrinus*), and black bears (*Ursus americanus*) as northern populations of these species colonized newly available habitat following glacial retreat. Coalescent-based analyses of molecular data can predict geographic patterns of genetic variation

in species that have experienced dramatic expansion from refugia (Fu 1997; Kuhner et al. 1998; Slatkin and Hudson 1991; Wakeley and Hey 1997). We predict that if wolves differentiated in separate refugia during ice ages and subsequently recolonized deglaciated regions, we should detect signals of expansion similar to those described for other high-latitude organisms (Rowe et al. 2004; Runck and Cook 2005).

Definitive investigations of population and phylogeographic structure require analyses of independent genetic loci, especially those that differ in mode of inheritance and rate of evolution. Comparing results of the maternally inherited mtDNA with those generated from highly variable autosomal microsatellite loci permits greater insight into historic and contemporary population parameters, while accounting for patterns associated with sex-biased dispersal. Here, we use sequence information from mtDNA to investigate wolves in North America to test the validity of the previously identified Coastal and Continental phylogroups in the Pacific Northwest uncovered through analyses of autosomal microsatellite loci (Weckworth et al. 2005). Additionally, these mtDNA analyses add an independent molecular perspective on populations described as distinct based on morphological characters (Fig. 1; Nowak 1995). Further, we test glacial refugia hypotheses and evaluate patterns of post-Pleistocene expansion to place phylogeographic patterns within a global context and to assess wolf colonization into North America from Eurasia.

MATERIALS AND METHODS

Sampling.—The sampling regime emphasized localities within Southeast Alaska and throughout northwestern North America (Fig. 1), including individual islands (REV) or island groups (KMW and POW) in the Alexander Archipelago; coastline of Southeast Alaska (MCN and MCS); interior Alaska (FAI, INT, CHA, and CHU); Kenai Peninsula of Alaska (KEN); Copper River Delta of southern coastal Alaska (CRD); British Columbia, Canada (BC); Yukon, Canada (YUK); and the Ural Mountains of Russia (RUS). FAI, INT, CHA, CHU, and YUK are in areas that were ice free during glacial maxima and thus represent populations that could have persisted in western Beringia. Wolves from 14 locations totaling 307 individuals were analyzed with populations assigned to 1 of 3 groups relative to geographic region: Continental (CNT), Coastal (CST), or Russian (RUS; Table 1). These include all 221 samples from prior microsatellite analyses (Weckworth et al. 2005). Furthermore, sequences obtained from GenBank from pre-extirpated populations in the conterminous United States were added to elucidate historic North American continental patterns of diversity (Leonard et al. 2005; Appendix I). Those sequences were from museum skins of wolves collected before 1917. We also included sequences representing localities throughout Eurasia (Appendix I). The sequences obtained from GenBank were used only in phylogenetic analyses, not population-level analyses.

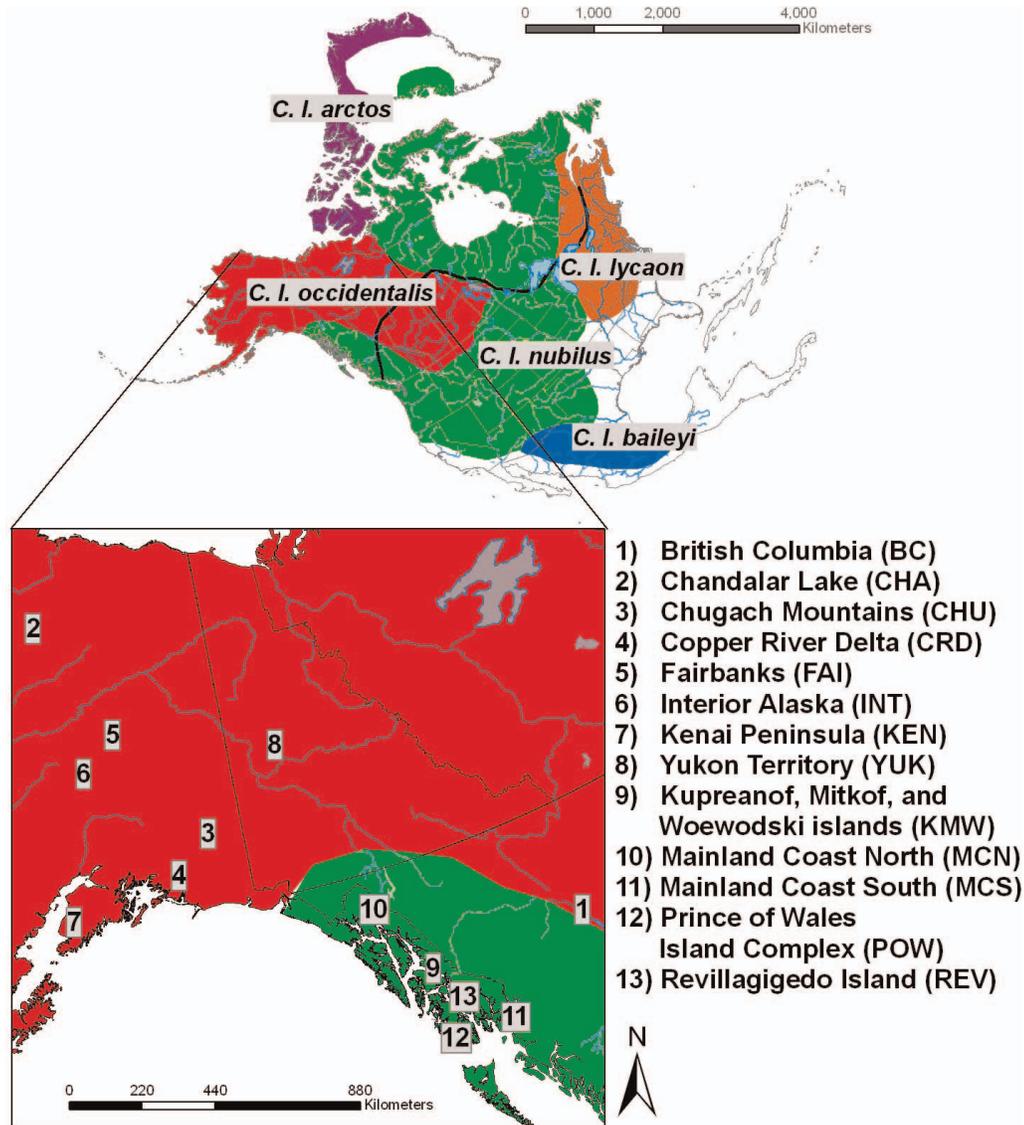


FIG. 1.—Map of North America (modified from Leonard et al. 2005) showing the pre-extirpation distribution of the gray wolf (*Canis lupus*) and the 5 subspecies based on morphological analysis (Nowak 1995). Solid black line marks the northern limit of eradication (Boitani 2003). Expanded inset of the Pacific Northwest indicates sampling locations and abbreviations.

The DNA was extracted from tissues (heart, spleen, skeletal muscle, skin, or blood) initially collected from hunters and trappers by the Alaska Department of Fish and Game and subsequently archived in collections of the University of Alaska Museum of the North, the Alaska Science Center, or the Museum of Southwestern Biology. Methods of DNA extraction followed Weckworth et al. (2005).

DNA sequencing.—We used approximately 611 nucleotide pairs (nt) comprising 94 nt of the 3' end of the wolf mtDNA cytochrome-*b* gene, the entire tRN^{thr} and tRN^{pro} genes (70 nt and 66 nt, respectively), and approximately 380 nt of the hypervariable portion I (Wakeley 1993) of the wolf mtDNA control region obtained using polymerase chain reaction. We used the primer pair C15243L (Kim et al. 1998; GenBank accession number U96639) and AH00019,

which was originally designed to anneal to the ursid conserved sequence block C (Talbot and Shields 1996).

Data were gathered in 2 laboratories: Idaho State University Department of Biological Sciences (ISU-DBS) and the United States Geological Survey Alaska Science Center Molecular Ecology Laboratory (ASC-MEL). Therefore, polymerase chain reaction, product purification, and sequencing techniques differed. Sequences gathered in the ASC-MEL followed universal tailed polymerase chain reaction, product cleanup, and sequencing protocols outlined in Jackson et al. (2008). Sequences gathered in the ISU-DBS followed polymerase chain reaction, product purification, and sequencing protocols outlined in Fleming and Cook (2002). Approximately 10% of the samples were subjected to polymerase chain reaction again and rerun to detect any sequencing errors.

TABLE 1.—Populations used in this study with their geographic group, abbreviation (Abbr.), sample size (n), number of haplotypes (k), haplotype diversity (h), nucleotide diversity (π), Fu's F_S value (F_S), P -values for F_S , Tajima's D (Taj. D), and P -values for Tajima's D .

Populations	Abbr.	n	k	h	π	F_S	F_S P -value	Taj. D	D P -value
Continental group	CNT	173	11	0.599	0.0072	2.097	0.806	0.921	0.806
British Columbia	BC	24	6	0.746	0.0099				
Chandalar Lake	CHA	3	1	0.000	0.0000				
Chugach Mountains	CHU	3	2	0.667	0.0014				
Copper River Delta	CRD	16	2	0.125	0.0018				
Fairbanks Quad	FAI	29	5	0.633	0.0087				
Interior Alaska	INT	29	3	0.310	0.0046				
Kenai Peninsula	KEN	57	3	0.451	0.0065				
Yukon	YUK	12	4	0.712	0.0076				
Coastal group	CST	130	4	0.122	0.0003	-3.772	0.003	-1.343	0.047
Kupreanof, Mitkof, and Woewodski islands, SE Alaska	KMW	23	2	0.087	0.0002				
Mainland Coast North, SE Alaska	MCN	5	1	0.000	0.0000				
Mainland Coast South, SE Alaska	MCS	10	2	0.356	0.0007				
Prince of Wales Island Complex, SE Alaska	POW	68	1	0.000	0.0000				
Revillagigedo Island, SE Alaska	REV	24	1	0.000	0.0000				
Russia	RUS	4	4	1.000	0.0125				
All		307	17	0.696	0.0075	0.022	0.588		

All sequences were compared to a reference sequence and other sequences from the population to edit ambiguities and verify mutations. All individuals generated at least 481 nt; in some instances the cytochrome-*b* portion and 36 nt of tRNA^{thr} did not amplify. For analyses sensitive to missing data, only the ubiquitous 481 nt were used. GenBank sequences (Appendix I) typically did not have cytochrome *b*, tRNA^{thr}, or tRNA^{pro}. However, these sequences all overlapped with the amplified portions of the control region described above.

Data analysis.—Phylogenetic relationships among haplotypes were examined using Bayesian methods. MODELTEST (Posada and Crandall 1998) was used to determine the simplest model of evolution for the data sets that was not significantly different from more complex models. Bayesian trees were created using MRBAYES version 3.0 (Huelsenbeck and Ronquist 2001); initiated with a random tree, 3 independent chains were run for 5 million iterations sampled every 1,000 generations. Log-likelihood values for each sampled generation were evaluated, and those preceding stationarity (plotting $-\ln L$ over generation time) were discarded. Data collected poststationarity were used to estimate posterior probability nodal support.

We used analyses of molecular variance (AMOVA—Excoffier et al. 1992) to test for significant geographic partitioning of a priori hypothesized genetic units, using ARLEQUIN (Schneider et al. 2000). This hierarchical analysis of variance partitions the total variance into covariance components due to differences among groups, among populations within groups, and within populations. These calculations were performed using pairwise distances, corrected using a model of evolution that best fit the data as identified using MODELTEST. Population pairwise estimates of Φ_{ST} were calculated and compared to pairwise F_{ST} values estimated from microsatellite data (Weckworth et al. 2005) via Mantel tests (Mantel 1967).

In addition to the a priori groups suggested by testable subspecies hypotheses applicable to wolves of the Pacific Northwest and Alaska (i.e., Hall and Kelson 1959; Nowak 1995), or broadscale divisions suggested by the analysis of microsatellite loci (i.e., Continental–Coastal designations—Weckworth et al. 2005), we experimented with various a posteriori groups in AMOVAs. A posteriori groups were suggested by the ambiguous placement of samples from British Columbia, analyses of nuclear DNA population trees, Bayesian analyses of population structuring, and those suggested by geographical isolation (see below and Weckworth et al. 2005). We assumed that the best geographic subdivisions were significantly different from random distributions and had maximum values of Φ_{CT} (for mtDNA data). Thus, if concordance exists between the distribution of genetic subdivisions at mtDNA and subspecies delineations, values of Φ_{CT} should be significant and larger than alternative groupings.

Population expansion was tested using multiple methods. Rapid population expansion leads to low levels of diversity among haplotypes over large areas (Hewitt 1996) and produces a basal polytomy or starlike phylogeny (Avise 2000). We used Tajima's D -test, a test of neutrality for which significant negative departures from 0 may indicate population expansions (Tajima 1989). Fu's F_S statistic is another neutrality test that is calculated from information on haplotype distributions. Significant negative Fu's F_S values, calculated in ARLEQUIN (Schneider et al. 2000), may indicate groups with recent population expansion (Fu 1997). Fu and Li's (1993) D^* and F^* statistics (calculated in DNASP 4.1—Rozas et al. 2003) can help distinguish background selection from population growth or range expansion when compared with Fu's F_S . If F_S is significant and D^* and F^* statistics are not, population growth or range expansion is indicated. Alternatively, the reverse situation suggests selection (Fu 1997). DNASP 4.1 also calculated the mismatch distribution of

TABLE 2.—Number of individuals per haplotype (Hap) found in each population (abbreviations follow Table 1). Haplotypes from Southeast Alaska Coastal populations are in boldface type.

Hap	BC	CHA	CHU	CRD	FAI	INT	KEN	YUK	KMW	MCN	MCS	POW	REV	RUS	Total
A	6	—	—	15	17	24	39	3	—	—	—	—	—	—	104
B	—	—	1	1	4	3	17	6	—	—	—	—	—	—	32
C	—	—	2	—	2	2	1	—	—	—	—	—	—	—	7
D	—	3	—	—	—	—	—	—	—	—	—	—	—	—	3
F	5	—	—	—	—	—	—	—	22	—	8	68	24	—	127
G	—	—	—	—	—	—	—	1	1	—	—	—	—	—	2
H	—	—	—	—	—	—	—	—	—	—	2	—	—	—	2
I	—	—	—	—	—	—	—	—	—	5	—	—	—	—	5
J	—	—	—	—	3	—	—	—	—	—	—	—	—	—	3
K	10	—	—	—	3	—	—	—	—	—	—	—	—	—	13
L	1	—	—	—	—	—	—	2	—	—	—	—	—	—	3
M	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1
N	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1
O	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
P	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
Q	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
R	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
Total	24	3	3	16	29	29	57	12	23	5	10	68	24	4	307

observed differences between pairs of haplotypes in 2 genetic groups, Continental and Coastal, previously identified using microsatellite loci (Weckworth et al. 2005). ARLEQUIN was used to calculate haplotype diversity (h), the probability that 2 randomly chosen haplotypes differ (varying from 0 to 1—Grant and Bowen 1998), and nucleotide diversity (π), the probability that 2 randomly chosen homologous nucleotides are different, which varies from 0 for no divergence to >0.10 for deep divergences (Grant and Bowen 1998). Finally, postglacial population expansion was tested using FLUCTUATE (Kuhner et al. 1998) version 1.4 for each geographic group, using the Metropolis–Hastings algorithm in a Markov chain Monte Carlo approach, to estimate demographic parameters. For each run, we used 10 short chains (sampling increments of 10; 1,000 steps), 10 long chains (sampling increments of 10; 20,000 steps), and a random starting tree. Replicates of each analysis were performed with different seed numbers to check for convergence in demographic parameter values. Exponential population growth is expected if g is determined to be greater than 0. Because these computations can show an upward bias (Kuhner et al. 1998), we used a conservative 99% confidence interval (99% CI).

RESULTS

Sequence diversity.—Of the 307 individuals sequenced, 17 haplotypes were identified (Table 2; GenBank accession numbers GQ376203–GQ376509). Four haplotypes (O–R) were restricted to RUS individuals, 2 haplotypes (H and I) were found only in the Coastal group, and 9 haplotypes (A–D and J–N) were found only in the Continental group. Two haplotypes were shared between the Coastal and Continental populations (F and G); haplotype F was predominantly found in the Coastal group, and haplotype G was found in 1 Coastal

and 1 Continental animal. Ten haplotypes were unique to an individual or population (Table 2). Overall, 2 haplotypes (A and F) accounted for nearly 76% of all individuals (104 and 127, respectively). BLAST analysis (Altschul et al. 1997) of the entire sequence failed to find identical haplotypes accessioned in GenBank, save 1 (G), which was identical at all 610 nucleotides compared with a haplotype sampled from Canada (GenBank accession number DQ480508—Björnerfeldt et al. 2006). Haplotype (h) and nucleotide (π) diversity were low for the Coastal group (0.122 and 0.0003, respectively). In contrast, the Continental group demonstrated high h and low π (0.599 and 0.0072, respectively).

Phylogeography.—MODELTEST identified HKY+I+G (Hasegawa et al. 1985) as the simplest evolutionary model that was significantly better than less complex models across all individuals. Parameters from this model were used in Bayesian analyses.

The unrooted Bayesian haplotype tree of individuals includes 4 phylogroups (Fig. 2) in North America and 1 in Eurasia. Sequences from pre-extirpated wolf populations in the conterminous United States (prefix ‘lu’ in Fig. 1) were added to our contemporary samples to provide a historic context. Phylogroup 1 is highly divergent and has strong nodal support (Fig. 2). The widespread Continental haplotype (A) was ubiquitous across all Continental populations except CHU and CHA (both small sample sizes from interior Alaska). Contemporary haplotypes in Phylogroup 1 (haplotypes A and M; Fig. 2) have a distribution corresponding to that of *C. l. occidentalis* (Fig. 1). However, the addition of the lu52 haplotype from a historical specimen collected in Oklahoma (Leonard et al. 2005) represents *C. l. nubilus* and is not consistent with the proposed taxonomy of Nowak (1995). Phylogroup 1 haplotypes occur within a larger cluster that also includes Phylogroup 2.

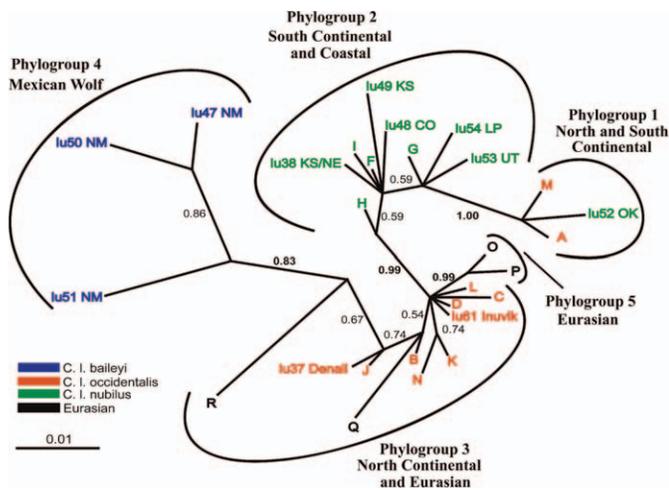


FIG. 2.—Bayesian tree of wolf haplotypes with log-likelihood values used to estimate posterior probability nodal support. Bracketing indicates 5 phylogroups for North American representatives and their geographic affiliations. Haplotypes are color coded according to haplotype locations and associated subspecies designation (Fig. 1). Haplotypes prefaced by “lu” are from extirpated southern populations (Leonard et al. 2005).

Coastal populations were restricted to Phylogroup 2, which included haplotype F, present in 122 of 130 Coastal individuals. This phylogroup also included 5 individuals from BC (haplotype F; Table 2) and 1 individual from YUK (haplotype G; Table 2). With the exception of the YUK individual, and possibly the BC individuals (collected at or near the boundary between *C. l. nubilus* and *C. l. occidentalis*; Table 2), samples were within the range of *C. l. nubilus* (Nowak 1995). The presence of haplotypes from extirpated southern populations (lu38, lu48, lu49, lu53, and lu54) in Phylogroup 2 indicates a wider geographic distribution of Phylogroup 2 through the conterminous United States (Fig. 2).

Phylogroup 3 included at least 1 individual from each of the Continental populations and 2 RUS haplotypes (R and Q) and were within the defined range of *C. l. occidentalis* (Fig. 1). Two RUS haplotypes (O and P) comprised a phylogroup (Phylogroup 5) that was sister to Phylogroup 3. Phylogroup 4 represents the endangered Mexican wolves (*C. l. baileyi*) and is monophyletic and diverged from other North American wolves (Fig. 2).

We included the highly diverged Himalayan (HW) and Indian wolves (IW), *C. l. chanco* (Sharma et al. 2003), to assess overall differentiation within a global sampling of wolves. This analysis also includes haplotypes from across the Eurasian continents (Fig. 3; Appendix I) to illuminate the magnitude of North America mitochondrial variation. Addition of those haplotypes indicates deeper genetic structuring across Eurasia, a finding consistent with the older fossil record of wolves in Eurasia (Kurtén 1968). Among haplotypes endemic to North America, Phylogroups 1, 2, and 4 (*C. l. baileyi*) are polyphyletic (Fig. 3; Appendix I).

Population structure.—When populations were divided into Coastal and Continental groups (Weckworth et al. 2005),

AMOVA results (Table 3, model A) indicate that 56.3% of all genetic variation distinguished geographic groups ($P < 0.0001$), 32.5% of variation was apportioned within populations ($P < 0.0001$), and 12.2% was relegated among populations within groups ($P < 0.0001$). Model A partitions the populations into 2 groups that correspond to the distributions of *C. l. nubilus* and *C. l. occidentalis* of Nowak (1995), thereby permitting a test of the validity of the 2 subspecies. Transferring BC from the Continental group (and from *C. l. occidentalis*) to the Coastal group (and into *C. l. nubilus*) results in a reduction of Φ_{CT} , although the value is still significant (Table 3, model B). The highest value of Φ_{CT} , however, was for model C, which tests subspecies designations applied by Hall and Kelson (1959) to wolves of the North Pacific coast and Alaska (e.g., *C. l. ligoni* describing wolves of the Alexander Archipelago and southeastern mainland, *C. l. pambasileus* describing wolves elsewhere in Alaska, and *C. l. columbianus* describing wolves of the Yukon and interior British Columbia). Pairwise estimates of Φ_{ST} are consistent with θ_{ST} in microsatellites and significantly correlated ($P = 0.0018$), with genetic distances highest between Coastal and Continental population comparisons (Table 4).

Population expansion.—No significant signature of expansion based on Fu’s F_S or Tajima’s D was observed for the Continental group. However, significant negative Fu’s F_S values ($P < 0.02$; Table 1), Tajima’s D ($P < 0.05$; Table 1), and nonsignificant D^* and F^* ($P > 0.05$; data not shown) were calculated in the Coastal group. These values corroborate h and π values for the Coastal group, suggesting deviation from neutrality as a result of either rapid population expansion or a selective sweep, and not the effects of background selection (Peck and Congdon 2004). A unimodal mismatch distribution also is consistent with demographic expansion in Coastal populations (Rogers and Harpending 1992), whereas in the Continental group the mismatch distribution was multimodal, consistent with demographic equilibrium or population substructuring (Fig. 4). Furthermore, growth estimates (g) calculated using FLUCTUATE yield values for the Coastal group that strongly support a recent population expansion ($g = 9,923$; 99% CI $\pm 1,203$). In contrast, the lower 99% CI of g for the Continental group encompasses 0 ($g = -30$; 99% CI ± 198), and thus does not support a history of expansion.

DISCUSSION

An mtDNA perspective on North American wolves that includes populations from coastal southeastern Alaska reveals a deep phylogeographic history of repeated exchange at the crossroads of the northern continents during glacial advances, in addition to northward colonization of wolves in North America following deglaciation at the end of the last glacial maximum. The addition of mitochondrial data from Southeast Alaska Coastal populations expands our view of contemporary diversity and population distinctiveness, previously inferred

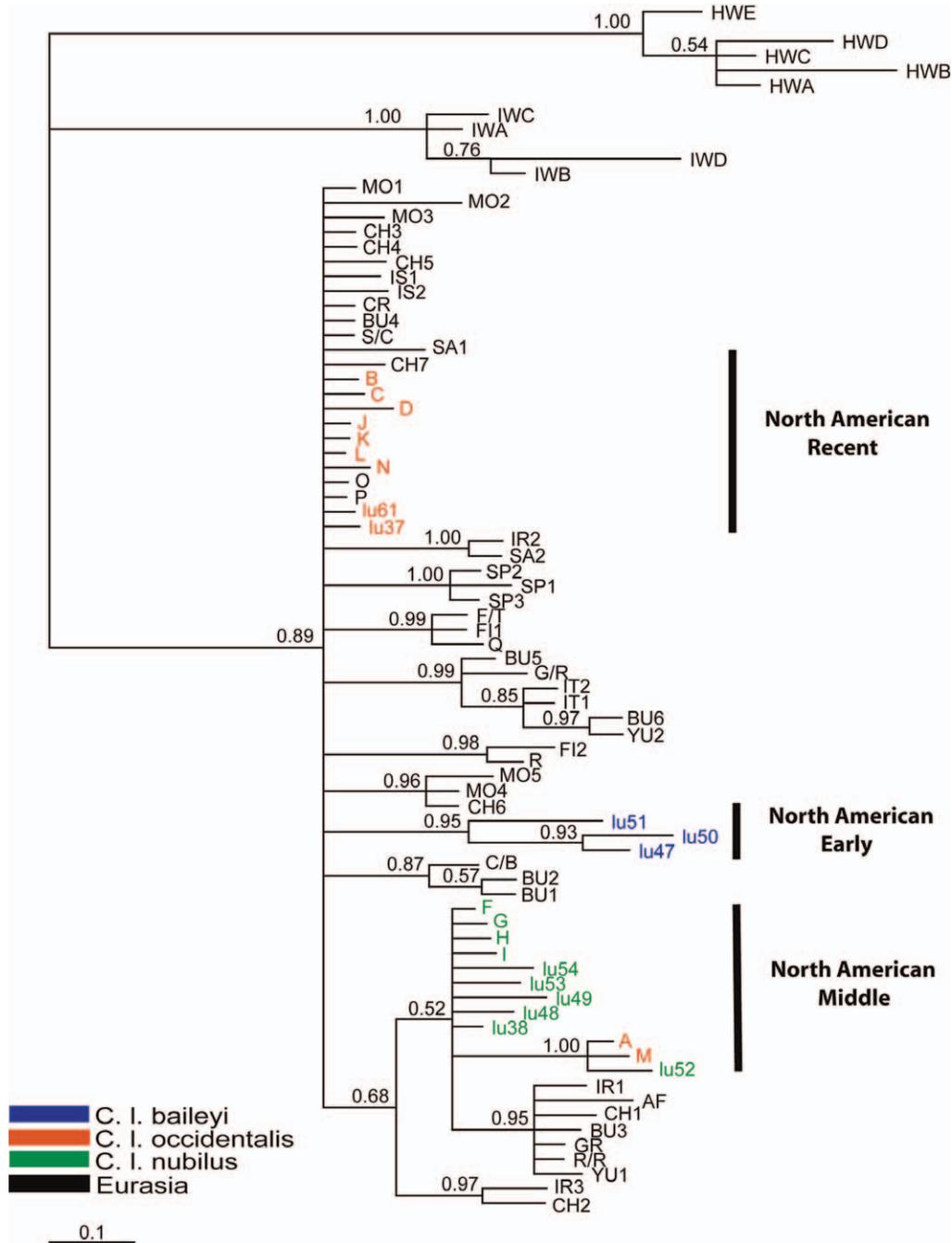


FIG. 3.—Bayesian tree of color-coded (same as Fig. 2) global haplotypes of wolves with log-likelihood values used to estimate posterior probability of nodal support. Bracketing indicates potential North American colonizations by representatives of 3 distinct phylogroups. Tree rooted with *Canis lupus chanco*, an ancient lineage of wolf (Leonard et al. 2005; Sharma et al. 2003).

TABLE 3.—Analyses of molecular variance (AMOVA) for hypothesized groupings based on sequence data for the mitochondrial DNA control region. Fixation indices are shown with the percent of total variance explained by the hypothesized regional grouping and its significance. The specifics of the models are described in the text.

Model	Hypothesized groupings	Φ_{SC}	Φ_{ST}	Φ_{CT}	% among groups	$P \Phi_{CT}$
A	[KMW, MCN, MCS, POW, REV] [BC, CHA, CHU, CRD, FAI, INT, KEN, YUK]	0.279	0.685	0.563	56.3	<0.001
B	[KMW, MCN, MCS, POW, REV, BC] [CHA, CHU, CRD, FAI, INT, KEN, YUK]	0.320	0.675	0.521	52.1	0.001
C	[KMW, MCN, MCS, POW, REV] [CHA, CHU, CRD, FAI, INT, KEN] [BC, YUK]	0.206	0.657	0.567	56.7	0.002

TABLE 4.—Pairwise estimates of microsatellite F_{ST} (lower matrix—Weckworth et al. 2005) and mitochondrial DNA Φ_{ST} (upper matrix) for all population pairs consistent between 2 studies. Significant comparisons ($P < 0.01$) are in boldface type. Matrices are significantly correlated (Mantel test, $P = 0.0018$). Population abbreviations follow Table 1.

	KMW	REV	POW	MCS	FAI	CRD	KEN	BC	YUK
KMW	—	0.00	0.06	0.15	0.59	0.93	0.64	0.44	0.72
REV	0.04	—	0.00	0.25	0.60	0.94	0.65	0.46	0.75
POW	0.20	0.10	—	0.47	0.74	0.97	0.75	0.63	0.87
MCS	0.06	0.02	0.14	—	0.49	0.88	0.59	0.32	0.58
FAI	0.22	0.16	0.22	0.12	—	0.19	0.01	0.13	0.09
CRD	0.18	0.11	0.17	0.12	0.07	—	0.11	0.45	0.58
KEN	0.19	0.13	0.23	0.13	0.10	0.12	—	0.28	0.23
BC	0.19	0.11	0.19	0.09	0.08	0.09	0.13	—	0.08
YUK	0.19	0.12	0.20	0.11	0.04	0.08	0.09	0.06	—

using data from the nuclear genome (Weckworth et al. 2005). Boreal species are often characterized by significant phylogeographic structure (Hewitt 2004), due in part to the dynamic glacial events of the Pleistocene (Weir and Schluter 2007). Examination of the data presented here demonstrates that even populations of highly vagile organisms such as wolves can show substantial phylogeographic structure. This general finding contrasts with earlier conclusions of limited phylogeographic structure in North American wolves (Roy et al. 1994;

Vilà et al. 1999) and demonstrates the value of extensive sampling, both temporally (Leonard et al. 2005) and spatially (Weckworth et al. 2005), and the importance of sampling regions of high biodiversity and complex biogeographic history such as Southeast Alaska.

Expansion and refugia.—Although phylogeographic structure characterizes boreal populations, minimal molecular variation is common within major phylogeographic groups for many boreal species (Demboski et al. 1999; Hewitt 2004). Coalescent-based perspectives of a limited set of boreal species reflect demographic expansion, with the signal most prominent in the deglaciated portions of a species range (Lessa et al. 2003). This pattern is predicted for populations that experienced dramatic expansion from refugial areas.

Episodes of rapid range expansion can have dramatic genetic consequences (Nichols and Hewitt 1994). Population expansion has been modeled as an advancing wave (Skellam 1951; Van den Bosch et al. 1988); however, some range expansions involve long-distance colonizations (pioneer colonization model—Nichols and Hewitt 1994) of vacant habitats, such as those available after the retreat of glacial ice (Coope 1990). Long-distance dispersal also may produce admixed populations when members of diverged, geographically distinct, source populations colonize the same region.

Wolves are capable of dispersing considerable distances and over sizable topographic and habitat barriers (Fritts 1983; Mech 1970, 1987). We suspect that populations in the Continental group are admixed because they are composed of lineages originating from the south (Phylogroup 1; Fig. 2) and from Beringia (Phylogroup 3; Fig. 2), although the possibility of incomplete lineage sorting cannot be dismissed without further tests. Lineages originating from southern refugia during the last glacial maximum (Phylogroups 2 and 4; Fig. 2) may be endemic to North America (Fig. 3) and likely the result of an earlier colonization of North America. Lineages representing a Beringian refugium (Phylogroups 5 and 3; Figs. 2 and 3) are closely allied with lineages found in Eurasia and likely represent a more recent colonization of North America, perhaps persisting through the last glacial maximum in Beringia. This hypothesis is supported by examining standard diversity metrics: the Continental group showed high haplotype diversity and low nucleotide diversity,

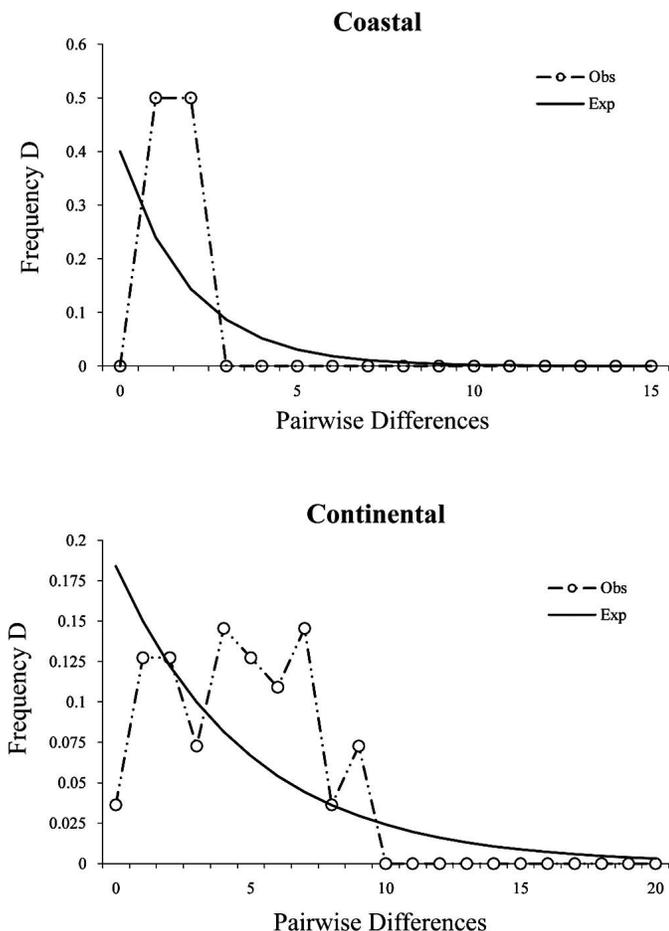


FIG. 4.—Mismatch distributions of observed differences between pairs of haplotypes for Coastal and Continental groups. Obs = observed distribution; Exp = expected distribution.

consistent with rapid growth from a small ancestral population. This pattern is expected if time has been sufficient for recovery of haplotype variation via mutation but is too short for the accumulation of large sequence differences (Avice 2000). However, mismatch distributions associated with the Continental group are multimodal, and growth estimates (g) were low (negative); these results are inconsistent with rapid population expansion from a single source. Assumptions related to our tests of expansion could have been violated by admixture that occurred in the Continental populations. A more critical test of a hypothesis of recent colonization of North America by ancestors of Phylogroups 5 and 3 would require much more extensive sampling of wolves in far eastern Siberia (i.e., western Beringia) and additional, independent loci.

In contrast to the Continental group, the low haplotype diversity for Coastal wolves may reflect a severe reduction in population size (Avice 2000). This observation, coupled with unimodal mismatch distribution and high positive growth estimates (g), suggests bouts of serial bottlenecking during rapid expansion. Similar scenarios suggested for other large mammals that persisted through glacial–interglacial cycles at high latitudes (Sage and Wolff 1986) may explain the low genetic diversity observed in some modern populations (Merilä et al. 1997; Milá et al. 2000), including Coastal wolves. Leonard et al. (2005) uncovered higher diversity in the extirpated southern populations (presumably refugial) of the conterminous United States, and these southern wolves have morphological (Nowak 1995) and genetic affinities to Coastal wolves (Figs. 1 and 2, respectively). Consequently, some of the genetic “legacy lost” (Leonard et al. 2005) through extirpation of southern wolves across the conterminous United States may persist in Southeast Alaska Coastal wolf populations. That these wolves now appear to be isolated from Continental populations (Weckworth et al. 2005) underscores the need for thoughtful management in this region.

Global phylogeographic patterns.—Wolves first appeared in the fossil record in Eurasia about 700,000 years ago (Kurtén 1968) and by the end of the Illinoian glaciation (~500,000 years ago) crossed into North America (Nowak 1979). Sequences obtained from GenBank allow spatially extensive comparisons (Fig. 3; Appendix I) and provide a more detailed view of the relationship between North American and Eurasian wolves. Significant phylogeographic breaks have been detected in central Siberia for other mammals (Galbreath and Cook 2004; Waltari et al. 2004), and the phylogeographic history of wolves appears to be complex and diverse in Asia (Aggarwal et al. 2007). In comparison, lower diversity within North American wolves supports the hypothesis of a more recent arrival of *C. lupus*. Previous molecular studies indicated historic movement of wolves between the continents via the Bering Land Bridge (Vilà et al. 1999).

Wolves in North America are not monophyletic, suggesting the possibility of multiple colonization events. Viewed in aggregate, the distribution of haplotypes into distinct phylogroups is consistent with potentially 3 such expansion events

from Asia (via the Bering Land Bridge) into North America. For example, Mexican wolves (*C. l. baileyi*) are deeply diverged from Eurasian and other North American lineages (Phylogroup 4; Figs. 2 and 3) and may be descendents of one of the earliest colonization waves of wolves into North America (Leonard et al. 2005). Phylogroups 1 and 2 (Fig. 2) are restricted to North America and could represent a 2nd expansion, but these clusters are not well supported in the Bayesian tree (Fig. 3). Phylogroup 3 is a mixture of Eurasian and North American haplotypes and could be a recent, or 3rd, expansion of wolves into North America. As wolves expanded south and eastward from the Beringian refugium following deglaciation, and southern refugial wolves expanded northward, long-distance dispersal across newly available habitat corridors might have facilitated widespread admixture of populations in western and central Canada. Contact and introgression are common in the Pacific Northwest as populations expanded into deglaciated regions from different refugia (Small et al. 2003; Runck et al. 2009). Rapid expansion of populations into recently deglaciated regions reflects the role of multiple refugia in structuring biotic diversity across the high latitudes (Hewitt 2004). An alternative explanation for the observed patterns is incomplete lineage sorting of this locus, so independent loci are required to test these alternative hypotheses.

Beringia not only formed a bridge for the exchange of organisms between Asia and North America during ice ages (Elias and Crocker 2008) but also served as a high-latitude refugium where species persisted and diverged during full glacial advances (Cook et al. 2005; Sher 1999). Previous phylogeographic studies of wolves have largely ignored the potential role of Beringia as a refugium and a potential site of diversification. A rigorous test of this set of colonization and diversification hypotheses will require more extensive sampling throughout Siberia (i.e., western Beringia).

Are Coastal wolves divergent?—Island populations in the Coastal group (Fig. 1) are almost entirely monotypic (haplotype F), but a few unique haplotypes also exist. The Coastal group is identified with Southeast Alaska, a landscape characterized by an extensive island archipelago (e.g., Alexander Archipelago) and a narrow strip of rugged coastline isolated from the remainder of North America by high coastal mountain ranges (see Fig. 1). Coastal refugia have been proposed in this region during periods of glaciation (Byun et al. 1997; Carrara et al. 2007), a hypothesis that is supported by the large number of endemic taxa along the coast (MacDonald and Cook 1996, 2007; Ogilvie 1989), including the distinctive molecular signature of insular populations of ermine (*Mustela ermine*—Fleming and Cook 2002). Phylogeographic studies are exploring these signatures of endemism (Cook et al. 2006; Dawson 2008; Talbot and Shields 1996), which appear enhanced by the highly fragmented and insular coastal landscape (Conroy et al. 1999; Lucid and Cook 2004).

Following glaciation, Southeast Alaska might have been repopulated by wolves moving northward along the coast (Pedersen 1982), as the glaciated Coast Range to the north and

northeast (Fig. 1) presented a significant physical barrier to access from southward-moving mammals from Beringia. Despite vagility of wolves, the Coast Range is recognized as a biogeographic barrier for large, mobile mammals (Klein 1965) and serves to restrict substantial movement out of Southeast Alaska to northern and eastern regions. Lack of genetic diversity, strong support for expansion, and the close evolutionary relationship between Coastal haplotypes and haplotypes from pre-extirpation specimens from the conterminous United States (Leonard et al. 2005) are consistent with a single colonization of wolves into the isolated coast of Southeast Alaska from a southern refugium (Klein 1965; Sage and Wolff 1986).

Several other mammalian species also show concordant geographic patterns of diversification. Nuclear and mitochondrial assessments of marten (*Martes Americana*—Small et al. 2003; Stone et al. 2002) and black bear (*U. americanus*—Peacock et al. 2007; Stone and Cook 2000) also identified genetically diverged Coastal and Continental clades. Black bear and marten expanded into Southeast Alaska following deglaciation of that region approximately 12,000 years ago (Small et al. 2003; Stone and Cook 2000). In contrast to the wolves, members of the Continental clade of marten and black bear recently have colonized the coast and now co-occur with Coastal forms in Southeast Alaska (Small et al. 2003; Stone and Cook 2000). Some Coastal haplotypes of wolves are found in low frequencies in adjacent Continental populations (YUK and BC) and perhaps reflect low levels of dispersal eastward along river corridors (e.g., Stikine River) out of Southeast Alaska. Dispersal events could have been historic, however, because the 6 Continental individuals that shared Coastal mtDNA haplotypes had nuclear profiles consistent with Continental populations. Additionally, dispersal events might have been primarily eastward as the common and widespread Continental haplotype (A) was not found in the Coastal group. Moreover, all Coastal haplotypes are restricted to a single clade, a pattern not found in other northern wolf populations. That signature further supports the more general discovery that a number of organisms inhabiting this coastal region are distinctive from their continental counterparts. In contrast, the lingering alpine glaciers of the Coast Range apparently prevented contemporary introgression of continental alleles into Coastal wolf populations. Furthermore, these Coastal populations contain remnants of the genetic diversity thought to have been lost with the extirpation of wolves in the contiguous United States to the south (Leonard et al. 2005). Preservation of this diversity is an important challenge for managers given the heavy human impact on these coastal wolves, their prey base, and associated habitat in Southeast Alaska (Person and Russell 2008).

Comparison with subspecies designations based on morphological data.—In North America examination of morphological data suggests as many as 5 subspecies of wolves stemming from the same number of refugial populations during the last glacial maximum (Nowak 1995). The affinities of wolves in interior British Columbia and Yukon (*C. l. nubilus* versus

C. l. occidentalis) are still unresolved (Fig. 1). Likewise, relationships among coastal British Columbia populations and southeastern Alaska Coastal populations are unclear. Muñoz-Fuentes et al. (2009) suggested that coastal British Columbia wolves are ecologically and genetically distinct from other North American wolf populations.

Wolf taxonomy and evolutionary history are complex and controversial in North America (Kyle et al. 2006; Murray and Waits 2007; Wheeldon and White 2009). Coastal populations currently are included in *C. l. nubilus*, a widespread subspecies that encompasses pre-eradication populations in the conterminous United States and eastern Canada (Nowak 1995). Nuclear (Weckworth et al. 2005) and mitochondrial data (Table 4) support the hypothesis that the Southeast Alaska Coastal wolves are distinctive and largely isolated from Continental populations. A morphological assessment described Coastal populations as an endemic subspecies (*C. l. ligoni*—Goldman 1944), a hypothesis that is consistent with the genetic data.

Conservation implications.—This study and others (Leonard et al. 2005; Weckworth et al. 2005) detected cryptic variation in North American wolves, substantially changing previous assertions suggesting minimal phylogeographic structure in these vagile carnivores. Uncovering the signature of complex historical events that impacted genetic variation across the landscape is central to interpreting how future climatic events will influence evolutionary change and ultimately critical to the effective management of diversity, particularly of those species of immediate conservation concern.

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APPENDIX I

Description of GenBank mitochondrial DNA sequences of *Canis lupus* used in phylogenetic analyses (Figs. 2 and 3). Listed by citation are haplotype designations used in figures and text, GenBank accession number, and geographic location of original sample.

Leonard et al. 2005.—1) lu47, AY812732, New Mexico, United States; 2) lu50, AY812735, New Mexico, United States; 3) lu51, AY812736, New Mexico, United States; 4) lu38, AY812731, Kansas/Nebraska, United States; 5) lu48, AY812733, Colorado, United States; 6) lu49, AY812734, Kansas, United States; 7) lu53, AY812738, Utah, United States; 8) lu54, AY812739, Labrador, Canada; 9) lu52, AY812737, Oklahoma, United States; 10) lu37, AY812730, Denali, Alaska, United States; 11) lu61, AY812741, Inuvik, Canada.

Savolainen et al. 2002.—1) CH1, AF530562, China; 2) CH2, AF530565, China; 3) CH3, AF530566, China; 4) CH4, AF530564, China; 5) CH5, AF530563, China; 6) CH6, AF530561, China.

Tsuda et al. 1997.—1) MO1, AB007379, Mongolia; 2) MO2, AB007375, Mongolia; 3) MO3, AB007376, Mongolia; 4) MO4, AB007377, Mongolia; 5) MO5, AB007378, Mongolia; 6) AF, AB007374, Afghanistan; 7) YU1, AB007373, Yugoslavia; 8) YU2, AB007372, Yugoslavia.

Vilà et al. 1999.—1) S/C, AF008142, Saudi Arabia and China; 2) SA1, AF008140, Saudi Arabia; 3) SA2, AF008141, Saudi Arabia; 4) G/R, AF008139, Greece and Romania; 5) IT1, AF008138, Italy; 6) SP1, AF008137, Spain; 7) R/R, AF008136, Romania and Russia; 8) CH7, AF008135, China.

Randi et al. 2000.—1) SP2, AF115702, Spain; 2) SP3, AF115703, Spain; 3) BU1, AF115688, Bulgaria; 4) BU2, AF115687, Bulgaria; 5) BU3, AF115691, Bulgaria; 6) BU4, AF115694, Bulgaria; 7) BU5, AF115701, Bulgaria; 8) BU6, AF115700, Bulgaria; 9) IT2, AF115699, Italy; 10) FI1, AF115692, Finland; 11) FI2, AF115698, Finland; 12) IS1, AF115697, Israel; 13) IS2, AF115696, Israel; 14) CR, AF115695, Croatia; 15) F/T, AF115693, Finland and Turkey; 16) GR, AF115690, Greece; 17) C/B, AF115689, Croatia and Bulgaria.

Sharma et al. 2003.—1) HWA, AY333738, Himalayas; 2) HWB, AY333739, Himalayas; 3) HWC, AY333740, Himalayas; 4) HWD, AY333741, Himalayas; 5) HWE, AY333742, Himalayas; 6) IWA, AY333743, India; 7) IWB, AY333744, India; 8) IWC, AY333745, India; 9) IWD, AY333746, India.

Unpublished.—1) IR1, AY570179, Iran; 2) IR2, AY570180, Iran; 3) IR3, AY570181, Iran.