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A Signal for Independent Coastal and Continental histories among North American wolves

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Abstract

Relatively little genetic variation has been uncovered in surveys across North American wolf populations. Pacific Northwest coastal wolves, in particular, have never been analysed. With an emphasis on coastal Alaska wolf populations, variation at 11 microsatellite loci was assessed. Coastal wolf populations were distinctive from continental wolves and high levels of diversity were found within this isolated and relatively small geographical region. Significant genetic structure within southeast Alaska relative to other populations in the Pacific Northwest, and lack of significant correlation between genetic and geographical distances suggest that differentiation of southeast Alaska wolves may be caused by barriers to gene flow, rather than isolation by distance. Morphological research also suggests that coastal wolves differ from continental populations. A series of studies of other mammals in the region also has uncovered distinctive evolutionary histories and high levels of endemism along the Pacific coast. Divergence of these coastal wolves is consistent with the unique phylogeographical history of the biota of this region and re-emphasizes the need for continued exploration of this biota to lay a framework for thoughtful management of southeast Alaska.

Keywords: *Canis lupus*, DNA, endemic, microsatellites, Pacific Northwest, southeast Alaska

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Introduction

The gray wolf (*Canis lupus*) has one of the most expansive natural ranges of any living mammalian species (Nowak 1979). In North America, *C. lupus* historically ranged from east to west coasts and from the Arctic Circle to central Mexico (Mech 1974). This extensive range is likely related to the wolf's ability to travel considerable distances (Mech 1970). Dispersal distances of up to 1000 km have been recorded for individual gray wolves, and typical dispersals may exceed 100 km (Fritts 1983; Mech 1987). In such a vagile species, geographical structuring should be minimal or, if present, reflect genetic structuring consistent with an isolation-by-distance model.

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The modern range of *C. lupus* includes southeast Alaska and the northern British Columbia coast, a landscape consisting of extensive islands (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). Phylogeographical studies in the region are beginning to uncover shared histories of colonization across a number of mammalian taxa (Talbot & Shields 1996; Cook *et al*. 2001; Lessa *et al*. 2003). The genetic patterns observed appear to be the result of postglacial mixing of refugial populations followed by differentiation because of the highly fragmented and insular nature of the landscape (Conroy *et al*. 1999).

Genetic analyses of *C. lupus* in North America have been extensive and have used multiple molecular markers across varying geographical scales including the Canadian Northwest (allozymes, Kennedy *et al*. 1991; microsatellites, Carmichael *et al*. 2001), the central Rocky Mountains (microsatellites, Forbes & Boyd 1997), eastern North America

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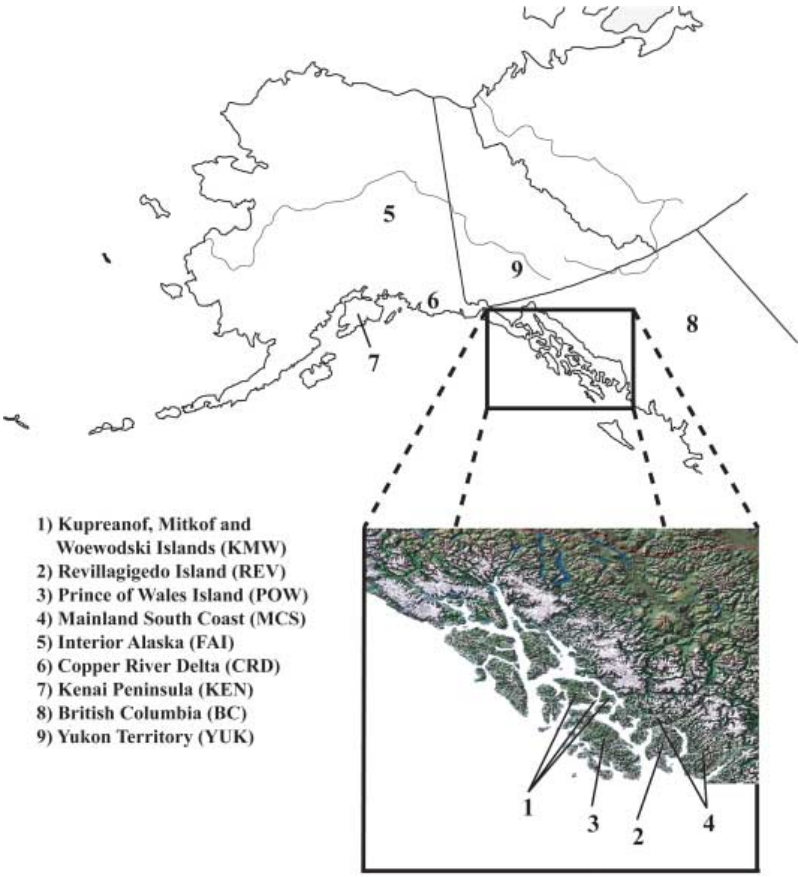

Fig. 1 Map of the Pacific Northwest with southeast Alaska expanded. Sampling locations and abbreviations are indicated.

(mtDNA sequences and microsatellites, Wilson *et al*. 2000), and North America (microsatellites, Roy *et al*. 1994; mtDNA sequences, Vilà *et al*. 1999). Analysis of mitochondrial DNA (mtDNA) control region in *C. lupus* indicated little historical variation in populations in North America, and suggests current low levels of variation may be because of recent restrictions to gene flow caused by fragmentation of habitat and population decline (Vilà *et al*. 1999). Microsatellite analysis of wolves across North America indicates divergence due to drift in finite populations and suggests this may have occurred in ice age refugia and that contemporary habitat fragmentation may be further contributing to higher levels of population differentiation (Roy *et al*. 1994). Previous studies encompassing island populations (Vancouver Island, Roy *et al*. 1994; Banks and Victoria Islands, Carmichael *et al*. 2001) have indicated moderate differentiation of island wolves from continental populations. However, none of the island systems previously investigated encompassed an area as large and geographically diverse as southeast Alaska. Here we lay a framework for interpreting the distinctiveness of coastal wolves, populations that may be increasingly vulnerable to harvest, loss of habitat, and loss of essential prey species (e.g. Person *et al*. 1996). Nuclear microsatellite loci are evaluated among and within wolf populations in the Pacific Northwest to assess geographical structure and levels of variation throughout the region. We begin to investigate the potential impact of episodic barriers and corridors related to the geological history of the region involving glaciers, changing sea levels, and geographical features that may promote isolation or contact between populations.

Materials and methods

Sampling

The sampling regime emphasized localities within southeast Alaska and throughout northwestern North America, including islands (Kupreanof, Mitkof, and Woewodski, KMW; Revillagigedo, REV; and Prince of Wales, POW) in the Alexander Archipelago, mainland southeast Alaska coast (MCS), interior Alaska (FAI), Kenai Peninsula of Alaska (KEN), Copper River delta of southern coastal Alaska (CRD), British Columbia (BC), and Yukon Territory (YUK). In southeast Alaska, populations were designated by biogeographical subregions (MacDonald & Cook 1996), with

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Table 1 Descriptive statistics for *Canis lupus* populations and clades

Populations	Abbr.	n	Alleles	Richness	H_E	H_O	F_{IS}	F_{ST}	M-ratio
Coastal group		101	5.00	3.21	0.52	0.48	0.05	0.12	0.795
Kupreanof, Mitkof, and Woewodski Islands, SE AK	KMW	26	3.73	3.00	0.46	0.43	0.08		0.747
Revillagigedo Island, SE AK	REV	24	4.09	3.46	0.57	0.59	−0.04		0.801
Prince of Wales Island, SE AK	POW	42	3.82	2.93	0.48	0.42	0.12		0.702
Mainland coast, SE AK	MCS	9	3.45	3.45	0.58	0.61	−0.04		0.737
Continental group		120	7.09	4.06	0.62	0.59	0.06	0.09	0.895
Fairbanks Quadrant, interior AK	FAI	29	5.55	4.62	0.64	0.59	0.08		0.846
Copper River Delta, coastal AK	CRD	14	3.64	3.43	0.58	0.53	0.10		0.737
Kenai Peninsula, coastal AK	KEN	33	3.55	3.17	0.55	0.55	0.01		0.750
British Columbia	BC	30	6.00	4.69	0.69	0.62	0.11		0.835
Yukon Territories	YUK	14	4.91	4.39	0.65	0.69	−0.06		0.825

Abbreviations (abbr.), sample size (n), mean number of alleles per locus (alleles), allelic richness (richness), expected heterozygosity (H_E), observed heterozygosity (H_O), F_{IS}, F_{ST} for the group comparison, and Garza & Williamson's (2001) M-ratios.

the exception of REV and the complex of KMW. These islands are within the same subregion, but were separated into two populations (mean distance between REV and KMW is 163 km). POW and mainland coastal (MCS) individuals each represent a separate subregion, resulting in a total of four designated populations in southeast coastal Alaska. Combined with five continental localities, a total of nine populations and 221 individuals were analysed (Table 1 and Fig. 1). Pack data were not available for most wolves, so whenever possible we avoided using tissues collected from the same latitude/longitude coordinates.

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 the University of Alaska Museum, the Alaska Science Center or Museum of Southwestern Biology. Methods of DNA extraction followed Talbot & Shields (1996) for muscle samples from KEN, Talbot *et al.* (in press) for blood samples from BC, and Fleming & Cook (2002) for all other tissue extractions.

Microsatellite genotyping

We screened 12 biparentally inherited dinucleotide repeat (CA) microsatellite loci known to be polymorphic in canids (Ostrander *et al*. 1993; Roy *et al*. 1994); 11 were found to be polymorphic and were used in subsequent analyses. Microsatellite loci were assayed using polymerase chain reaction (PCR) with primers end-labelled using IRDye 700 and 800 fluorescent tags (LI-COR). PCR amplifications were carried out in a final volume of 10 μL on a Robocycler (Stratagene) and contained 2–100 ng of genomic DNA, 0.2 mM of dNTPs, 3.6–3.9 pmoles of unlabelled forward primers, 4.0 pmoles of unlabelled reverse primer, 0.1–0.4 pmoles (depending on locus) of IRD-labelled primer, 0.1 μg of BSA, 1X PCR buffer (Perkin-Elmer Cetus I), and 0.5 units of Ampli*Taq* DNA polymerase (PE Biosystems). Reactions typically began with 94 °C for 2 min and continued with 40 cycles each of 94 °C for 1 minute, 50–56 °C for 1 minute, and 72 °C for 1 minute. A 30-minute extension at 72 °C concluded each reaction. The fluorescently labelled PCR products were electrophoresed on a 48- or 64-well 6% polyacrylamide gel, on a LI-COR 4200 L-2 LR automated sequencer. Initially, 24 individuals were scored against a fluorescently labelled M13 sequence ladder of known size (Amersham Pharmacia Biotech). Two or three individuals heterozygous at each locus were selected among the 24 sized individuals and included in all subsequent genotyping gels as unambiguous size standards typically occupying 9–15 lanes. Microsatellite fragment data were captured using LI-COR GENE IMAGEIR DATA ANALYSIS software. For quality control purposes, a minimum of 10% of individuals were randomly selected for each locus, re-extracted from the original tissue source, and subjected to PCR amplification.

Data analysis

Allele number and heterozygosity (observed and expected) for each locus across populations were calculated using MSA 3.0 (Dieringer & Schlötterer 2003). Allelic richness (Petit *et al*. 1998) per locus and population and fixation indices of heterozygosity (F_{IS}; Hartl & Clark 1997) were calculated using FSTAT 2.9.3 (Goudet 2001). GENEPOP version 3.3 ftp://isem.isem.univ-montp2.fr/pup/pc/genepop; Raymond & Rousset 1995) was used to test for genotypic linkage disequilibrium (LD) and Hardy–Weinberg equilibrium (HWE). Deviations from LD and HWE were tested between each pair of loci for each population and per locus, respectively. For loci with four or fewer alleles, exact tests (Louis & Dempster 1987) were used to estimate P values to test for deviations from HWE. For loci with more than four

alleles, estimated *P* values used the Markov chain method (Guo & Thompson 1992). Genotypic LD was tested using the Markov chain method with 10 000 dememorizations, 5000 batches and 10 000 iterations. *P* values for tests were corrected using a strict Bonferroni adjustment (initial α = 0.05) for multiple comparisons. Pairwise estimates of population differentiation using allelic frequency (F_{ST}) were calculated to generate estimates of gene flow [$M : M = (1/F_{ST} - 1)/4$] (Slatkin 1993). Isolation-by-distance analysis (Slatkin 1993) was performed plotting the log of geographical distances between pairs of populations vs. the log of *M*. A Mantel test (Mantel 1967) was used to assess the significance of the correlation between these variables using 10 000 permutations of the matrix computed through the ISOLDE subroutine. Latitude and longitude coordinates for each individual were averaged for each population and the geographical distance was measured as the straight-line length between each population's average latitude/longitude coordinate.

Populations were assessed for evidence of a recent reduction in population size using the program BOTTLENECK (Piry *et al*. 1999). Populations that have experienced a recent genetic bottleneck exhibit a correlative reduction of allele numbers and heterozygosity at polymorphic loci. However, allelic numbers are reduced faster than gene diversity. Thus, a recently reduced population is characterized when observed heterozygosity is larger than expected equilibrium heterozygosity, which is calculated from the observed number of alleles under the assumption of a constant size population (mutation-drift equilibrium) (Cornuet & Luikart 1996). Most microsatellite data sets have been shown to fit a two-phase model of mutation (TPM), rather than the infinite allele model (IAM) or stepwise mutation model (SMM) (Di Rienzo *et al*. 1994). Thus, our analysis was conducted using a TPM with multistep mutations accounting for 5%, 10%, 20%, or 30% of all mutations. A Wilcoxon signed rank test was used to determine which populations have a significant number of loci with gene diversity excess (Luikart *et al*. 1998). This statistical test was the most appropriate because majority of our populations are represented by fewer than 30 samples. Genetic evidence for historic bottlenecks using microsatellite loci can consist of gaps in the size distribution of alleles. The incompleteness of these distributions can be quantified as the M-ratio, the mean ratio across all loci of the number of alleles to the allele size range (Garza & Williamson 2001). Calculation of M-ratios is dependent on the loci following a pattern of mutation where changes in allele state consist of decreasing or increasing numbers of repeats. Loci with single base-pair differences cannot be used. Mean of M-ratios were calculated for each population using AGARST version 2.9 (Harley 2002). Contrary to BOTTLENECK, a declining M-ratio after a population is reduced in size is largely independent of any mutation process because drift or migration would play a larger role than mutation in accrual of new alleles postbottleneck (Garza & Williamson 2001).

PHYLIP version 3.6 (http://evolution.genetics.washington.edu/phylip.html; Felsenstein 1993) was used to calculate pairwise chord distances (Cavalli-Sforza & Edwards 1967) among populations and population networks using the allele frequency matrix created in GENEPOP. Chord distances were calculated for all population pairs in the subroutine GENDIST and used to construct a maximum-likelihood tree (ML) in the subroutine CONTML. To test the strength of the tree topology, 1000 bootstrap replicates were generated in the SEQBOOT subroutine and analysed in GENDIST. Tree topologies were created for all replicates in CONTML and a consensus tree was generated in the subroutine CONSENSE. Tree files were viewed using TREEVIEW version 2.0 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html; Page 1996).

A Bayesian-clustering program utilizing a Markov chain Monte Carlo (MCMC) approach, STRUCTURE version 2 (http://pritch.bsd.uchicago.edu; Pritchard *et al*. 2000), was used to conduct admixture and assignment tests and examine population structure according to inferred population clusters based on multilocus genotype data. We calculated the probability of individual assignments to population clusters (K). A series of tests was performed using different numbers of population clusters (MAXPOPS 1–20) to guide an empirical estimate of the number of identifiable populations (Table 2). The probability of how the data best fit into each number of assumed clusters was estimated in each case (ln probability of the data) without using any prior population information, so that individuals were assigned to a cluster based solely on their multilocus genotypic profile. Burn-in and replication values were set at 100 000 and 1 000 000, respectively, and each

Table 2 Proportion of membership (above 0.01) of each population in each of the seven clusters, given no prior information of population origin using STRUCTURE (Pritchard *et al*. 2000) and assignment to one of two clusters assumed to be either Coastal (CST) or Continental (CNT) group. Highest proportion of membership assigned to a single cluster is in bold type. Population (Pop) abbreviations as in Table 1

	Clusters								
Pop	1	2	3	4	5	6	7	CST	CNT
KMW	0.01	0.10	0.02	0.03	0.01	0.02	**0.83**	0.96	0.04
REV	0.02	0.21	0.02	0.05	0.03	0.02	**0.64**	0.96	0.04
POW	0.01	**0.83**	0.02	0.04	0.02	0.01	0.07	0.95	0.05
MCS	0.02	0.04	0.04	0.10	0.02	0.04	**0.75**	0.92	0.08
FAI	**0.38**	0.01	**0.38**	0.16	0.04	0.01	0.01	0.04	0.96
CRD	0.13	0.06	0.16	**0.27**	0.18	0.09	0.12	0.23	0.77
KEN	0.01	0.01	0.01	0.02	**0.92**	0.01	0.02	0.07	0.93
BC	0.06	0.01	0.09	0.20	0.03	**0.60**	0.01	0.03	0.97
YUK	**0.39**	0.01	0.19	0.26	0.04	0.06	0.04	0.03	0.97

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test yielded a log-likelihood value of the data (ln probability), with the highest indicating which test was closest to the actual number of genetically distinct populations. Individuals were assigned probabilistically to a population or to multiple populations if their genotype profile indicated admixture.

ARLEQUIN (Schneider *et al*. 2000) was used to conduct an analysis of molecular variance (AMOVA, Excoffier *et al*. 1992). AMOVA partitions the total variance into covariance components due to differences among groups, among populations within groups and among individuals. These calculations were performed using allele frequency data (F_{ST}; Excoffier *et al*. 1992). The nine populations were divided into a southeast coastal group (Coastal) and a continental group (Continental), to define a particular genetic structure to test.

Results

Microsatellite variation

Number of alleles per locus across all populations ranged from two (locus *C172*) to 15 (locus *C30*), with an average of 7.5 alleles (Appendix). Values of expected heterozygosity (H_E) averaged across loci ranged from 0.46 (KMW) to 0.69 (BC). Mean number of alleles per locus (observed allelic diversity) ranged from 3.45 (MCS) to 6.0 (BC) among populations, and was 5.00 and 7.09 in the Coastal and Continental groups, respectively. Continental populations had a higher frequency of private alleles than southeast Coastal populations (4.6 vs. 1.25 alleles per population, respectively). Of the alleles unique to the southeast Coastal region, none was widespread; each was unique to a single individual. In contrast, 12 alleles unique to Continental regions were found in at least two different populations. Allelic richness (Petit *et al*. 1998) was highest in BC (4.69) and lowest on POW (2.93). Population specific alleles were observed in five populations (KMW, REV, POW, FAI, and BC; see Appendix), however, in southeast Coastal populations, these alleles were restricted to a single individual. In Continental populations, private alleles occurred in one to five individuals. Southeast Coastal and Continental groups were not significantly different in allelic richness ($P = 0.093$).

Exact tests of genotypic LD indicated that *C030* and *C250* were associated in FAI population. Globally, *C030* is associated with both *C213* and *C250*. However, for each of these associations the loci have been mapped to different chromosomes (Breen *et al*. 2001), indicating that the LD observed is not due to physical linkage. Fixation indices (F_{IS}) averaged for each population across all loci had high and low values of 0.12 and −0.06 for POW and YUK, respectively (not significantly different from zero, Table 1). Significant departures from HWE were found in loci *C123* and *C203* in MCS and FAI, respectively. When a global test across loci and across populations was performed, the null hypothesis of equilibrium was rejected ($P < 0.001$, $\alpha = 0.05$), however, after correcting for multiple tests, the null hypothesis may not be rejected ($\alpha = 0.0004$). Observed hetero-zygosity and fixation index values did not differ significantly between southeast Coastal and Continental groups ($P = 0.118$ and 0.859 for H_O and F_{IS}, respectively, Table 1).

Geographic variation and structuring

We used chord distances for ML assessment of genetic structure. The unrooted network of chord distances among populations identified two well-defined clusters (bootstrap value of 95% in ML; Fig. 2). Group 1 consists of all southeast Coastal populations, and group 2 was all Continental populations. There were also moderate levels of support for geographical structuring of CRD and KEN populations (54% and 60%, respectively). Geographic structuring was also evident in group 1, where moderate levels of support indicate geographical partitioning (KMW and MCS at 74%, POW at 58%).

Using STRUCTURE, we tested for the number of populations that best described the distribution of the data into population clusters (K). Our data were sampled from nine designated locations (Table 1), but the highest probability of the data (ln = −5593.6) was found with clusters set at seven

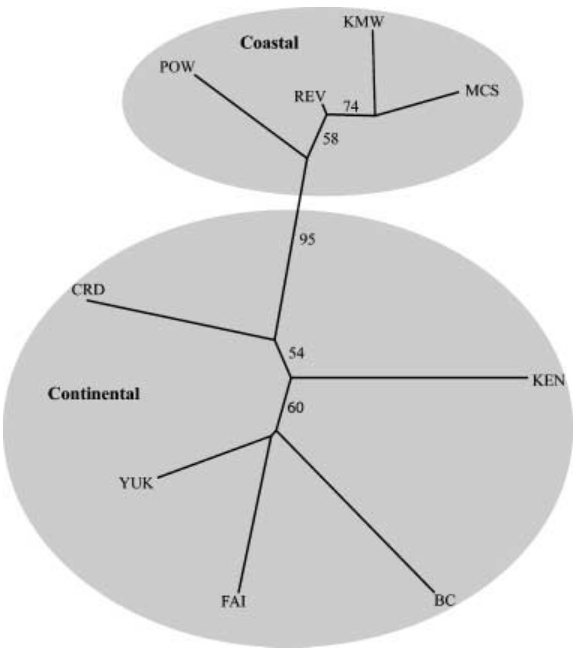

Fig. 2 Unrooted maximum-likelihood tree (population abbreviations from Table 1) showing bootstrap values > 50%.

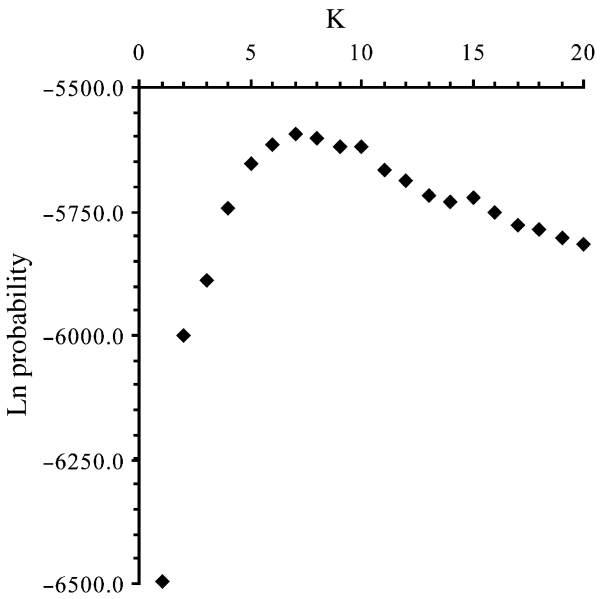

Fig. 3 Bayesian clustering analyses for all 221 individuals analysed at 11 microsatellite loci. Individuals were assigned to clusters using STRUCTURE (Pritchard *et al*. 2000) without using prior information of population origin. Ln probability (*y*-axis) of being assigned to 1 through 20 clusters (K, *x*-axis) indicates most probable number of distinct populations for individuals analysed.

(Fig. 3). The presence of seven populations was inferred entirely based on multilocus microsatellite genotypes.

Additional assignment tests were performed using STRUCTURE, to test how well individuals fit into predefined groups. The results of this analysis can be visualized in a colour histogram where each of the predefined clusters is represented by a different colour and the proportion of each individual assigned to each cluster is made up of each colour. Individuals belonging to a discrete population are assigned to one cluster and will be a single colour. Admixed individuals are identified by having multiple colours. The assignment of individuals to specific clusters was sometimes indicative of discrete or nearly discrete populations and regions (Fig. 4a,b, Table 2). An assignment test was conducted without prior information of the population origin of each individual, with clusters set to seven, to examine how well the distinctiveness of clustering mirrored the STRUCTURE analysis of K. Six populations, including all Coastal populations, had 60% or more of their membership assigned to a single cluster, and all clusters contained some proportion of membership from every other population (Fig. 4, Table 2). As the histogram displays, three populations – KEN, POW, and KMW – have individuals assigned with a large proportion to one colour (i.e. cluster; Fig. 4) with 92%, 83%, and 83% of the allele frequency profiles of individuals assigned to a single cluster, respectively (Table 2). Within Coastal populations, individuals were assigned primarily to one of two clusters, one associated primarily with POW or another associated with KMW, MCS, and REV (see Fig. 4 and Table 3) suggesting isolation of POW from the rest of southeast Alaska. To test for assignment into a distinct group, a second assignment test was performed with number of clusters set at two, representing the Coastal and Continental groups (Fig. 2). A clear distinction of Coastal from Continental is evident in the histogram (Fig. 4B). With one exception (CRD), the mean assignment of individuals of each population was > 90% membership in the appropriate cluster (Table 2). Twenty-three percent of individuals within CRD were assigned membership in the Coastal cluster (Table 2), suggesting admixture between Coastal populations and CRD. CRD was also the most poorly assigned population, with no greater than 27% assignment to any one population cluster (Table 2). CRD may represent individuals that are highly admixed, or contain individuals from populations not included in this study. Overall, however, the assignment test strongly supports the presence of two primary groups (Coastal and Continental).

Estimates of gene flow and genetic distances

Mean distance between Coastal and Continental populations was 903 km. Mean distance within Coastal and Continental populations was 108 and 814 km, respectively. No pattern of isolation by distance is evident when log of M is plotted against log of geographical distance between pairs of populations (Fig. 5). Mantel tests showed no significant correlation between pairwise estimates of log M and log geographical distance ($P > 0.05$), suggesting restricted gene flow caused by geographical distance alone does not

Table 3 AMOVA results for *Canis lupus* data using ARLEQUIN (Schneider *et al*. 2000). Populations were assigned into two groups identified by geographical association (Coastal and Continental). F_{ST} AMOVA used allele frequency differences as the distance measure

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)	Fixation indices	*P* value
Among groups	1	88.9	0.30	8.04	$F_{CT} = 0.080$	< 0.01
Among populations within groups	7	135.6	0.35	9.37	$F_{SC} = 0.102$	< 0.01
Within populations	433	1330.9	3.07	82.59	$F_{ST} = 0.174$	< 0.01
Total	441	1555.4	3.72			

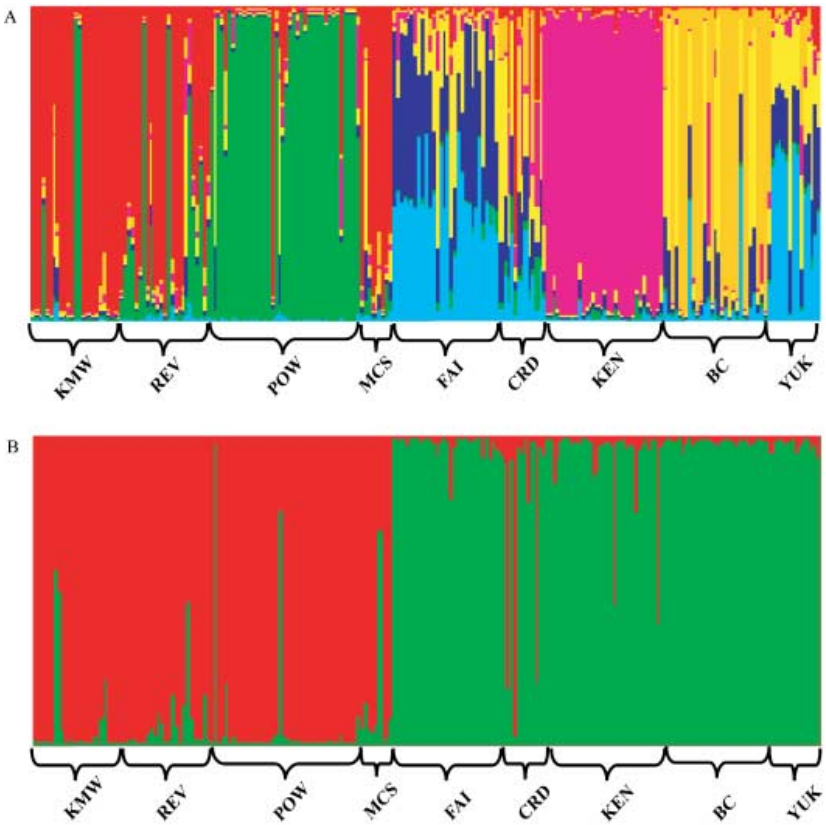

Fig. 4 Histograms of STRUCTURE assignment tests. Each vertical bar represents an individual and its assignment proportion into one of seven (A) or two (B) clusters. More than one colour per individual indicates admixture. Individuals are arranged in order by populations; abbreviations follow Table 1.

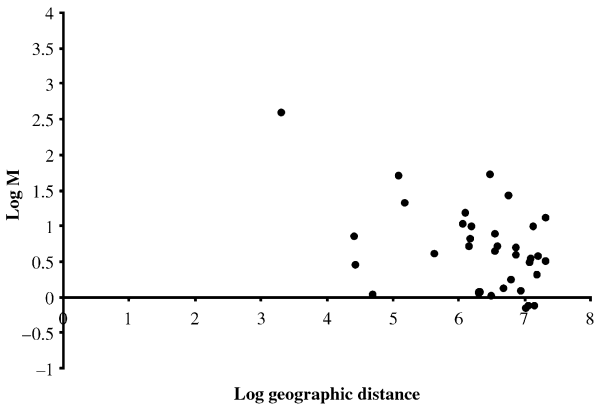

Fig. 5 Relationships between pairwise geographical distances and estimates of gene flow (M) based on F_{ST} for microsatellites.

Table 4 Pairwise estimates of F_{ST} genetic distances for all population pairs. Population abbreviations as in Table 1

	KMW	REV	POW	MCS	FAI	CRD	KEN	BC
REV	0.04							
POW	0.20	0.10						
MCS	0.06	0.02	0.14					
FAI	0.22	0.16	0.22	0.12				
CRD	0.18	0.11	0.17	0.12	0.07			
KEN	0.19	0.13	0.23	0.13	0.10	0.12		
BC	0.19	0.11	0.19	0.09	0.08	0.09	0.13	
YUK	0.19	0.12	0.20	0.11	0.04	0.08	0.09	0.06

account for the genetic differences between populations. When dispersal is restricted, the absence of a pattern of isolation by distance is an indicator of nonequilibrium, suggesting that the current distribution of the species has resulted from recent colonization (Slatkin 1993).

When populations were divided into Coastal and Continental groups, the AMOVA tests for the significance of microsatellite genetic variability between groups was similar to the calculated F_{ST} (Table 3, F_{CT} = 8.04%, $P < 0.01$). Coastal and Continental groups differ significantly in variance in allele frequencies (F_{ST}).

Pairwise comparisons of F_{ST} were used to evaluate genetic distances within and between each of the two groups (Table 4). Mean distances between groups were nearly twice that of mean distances within groups. Statistical tests for F_{ST} showed that mean genetic distances between the two groups are significantly different from distances between Continental populations ($P < 0.05$). F_{ST} values indicate that Coastal populations are distinctive from Continental populations. Greatest significant distance was between POW and KEN (F_{ST} = 0.23). The lowest genetic distance between

Coastal and Continental populations was between BC and MCS (F_{ST} = 0.09). Within Coastal group, the highest F_{ST} value (0.20) was observed between POW and KMW populations. The greatest distance within Continental populations was between KEN and BC (F_{ST} = 0.13).

Mean F_{ST} distance among Coastal populations was greater than mean distance among Continental populations, which encompass a much greater geographical area. This finding likely reflects the highly fragmented and insular nature of the coastal region. In sum, mean genetic distances among populations within Coastal and Continental regions differ, but these differences are not significant ($P > 0.05$). Overall, distances are greatest between the two groups.

Population bottlenecks

The MCS population was not analysed using BOTTLENECK because of small sample size ($n = 9$). Wilcoxon tests of significance were consistent across the four TPM scenarios used. After correcting for multiple tests, significant excess heterozygosity (one-tailed Wilcoxon test for H excess) was detected in only KEN populations with a TPM of 20% and 30% ($P = 0.0034$ for both). Significance decreased as TPM converged to a purely stepwise mutation model. M-ratios for populations varied between 0.702 (POW) and 0.846 (FAI), and calculations for groups yielded 0.795 and 0.895 for Coastal and Continental, respectively (Table 1). In comparison, a historically stable group of wolves in North America (Roy *et al*. 1994) yielded M-ratio = 0.858, and the reduced population of Mexican wolves (Garcia-Moreno *et al*. 1996) yielded M-ratio = 0.647 (from Table 2 in Garza & Williamson 2001). M-ratios do not support a historic bottleneck for any population analysed.

Discussion

Since Swarth's (1936) characterization of the Sitkan District, the North Pacific coast has been recognized as a distinctive biogeographical region in North America (Klein 1965; Cook & MacDonald 2001). Phylogeographical assessments of a suite of mammals have uncovered previously undetected endemism (e.g. Talbot & Shields 1996; Demboski *et al*. 1999; Conroy & Cook 2000; Stone *et al*. 2002). Glacial cycles of the late Pleistocene created a dynamic history of isolation and fragmentation in the Pacific Northwest (Pielou 1991), and this geological history apparently played a significant role in evolution and divergence (e.g. Small *et al*. 2003). Distinctive coastal and continental lineages have been identified in a variety of northwestern terrestrial mammals, covering a multitude of life histories from the dusky shrew (*Sorex monticolus*) to black bears (*Ursus americanus*) (Cook *et al*. 2001). Similarly, coastal gray wolves appear to have experienced a distinctive evolutionary history from continental populations. Assignment tests and networks based on chord distances are consistent with geographical isolation and distinction of southeast Coastal wolves from adjacent Continental populations. The relatively divergent population structure found within southeast Alaska further supports these ideas, but samples from coastal populations in British Columbia and regions south of southeast Alaska need to be examined to effectively test the hypothesis of a coastal/continental split.

Genetic distances among populations of *Canis lupus* were independent of geographical distance, suggesting that either dispersal distances are sufficiently large to confound genetic differentiation or that barriers to dispersal are more important in structuring genetic variation in wolves than is geographical distance (Slatkin 1993; Roy *et al*. 1994). *F*-statistics, tests of HWE, allelic diversity, and levels of heterozygosity further suggest that although these populations have been isolated, they have maintained relatively high allelic diversity and display identifiable geographical structuring within a comparably small geographical area.

Fossil record indicates that *C. lupus* migrated from Eurasia to North America approximately 500 000 BP, during the late Pleistocene (Nowak 1979). Morphological analyses of skull features suggest as many as five subspecies of wolves in North America (Nowak 1995). During the most recent ice age (ending about 10 000 BP in North America), *C. lupus* persisted in two or more refugia, with southern continental United States, Arctic Canada, and eastern Beringia (Alaska) suggested as possibilities (Nowak 1983, 1995). Of these five subspecies, our sampling regime includes two, *Canis lupus nubilus* and *Canis lupus occidentalis*. *C. l. nubilus* encompasses southeast Alaska, western British Columbia, much of the contiguous United States and eastern Canada; while *C. l. occidentalis* includes western Canada and the rest of Alaska (Nowak 1995). The molecular perspective developed in this study does not coincide with the current morphological scheme. The original morphological classification of wolves included three subspecies along the North Pacific coast: *Canis lupus alces* of the Kenai Peninsula of Alaska, *Canis lupus crassodon* of Vancouver Island, British Columbia, and *Canis lupus ligoni* of southeast Alaska (Goldman 1944). The latter subspecies corresponds to our Coastal populations.

Gray wolves in southeast Alaska are hypothesized to be postglacial colonizers from one or more southern refugia. Fossil evidence of wolves has not been found in the Alexander Archipelago, representing one of the few extant species on the islands that has not been identified in extensive palaeontological excavations centred in the southern Alexander Archipelago (Heaton, personal communication). Furthermore, no diagnostic alleles were observed in southeast Coastal wolves. Viewed in aggregate, this information suggests a Holocene colonization of the region by wolves. Klein (1965) suggests that these wolves followed the black-tailed deer from southern regions, north, into southeastern Alaska after the last glacial advance. The distribution of the

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coastal lineage of black bears in the Pacific Northwest is similar to distribution of these coastal wolves. Coastal bears likely colonized the region from a western refugium (or refugia) south of the Pleistocene ice sheets (Klein 1965; Byun *et al*. 1997; Wooding & Ward 1997; Stone & Cook 2000). Alternatively, wolves in southeast Alaska may have colonized the coast southward from Beringia as has been hypothesized for brown bear (*Ursus arctos*; Pasitschniak-Arts 1993; Waits *et al*. 1998). High levels of variation in the coastal wolves and significant genetic distance from populations adjacent to southeast Alaska may have resulted from not one, but multiple colonization events of southeast Alaska from different sources. Small *et al*. (2003) proposed that populations of marten (*Martes americana*) colonized northward along the coast from a southern refugium 10 000–12 000 BP, following the recession of the ice sheets and establishment of forest habitat. Rising sea level may have then isolated founders on various islands of the Alexander Archipelago and Haida Gwaii (Warner *et al*. 1982; Fedje & Josenhans 2000). Subsequently, members of the continental clade of marten (*Martes americana americana*) colonized southeastern coastal Alaska from east of the Coast Range. A similar hypothesis is presented for black bears across the same region (Wooding & Ward 1997; Stone & Cook 2000). The distribution of wolves in the Pacific Northwest coincides with that of black bears, and these large carnivores may have followed similar colonization routes (Klein 1965). Unlike black bears, however, only the coastal lineage of wolves is found in southeast Alaska. Wolves that prey on deer tend to have higher population densities than wolves preying on other ungulates (Person *et al*. 2001). Deer are the primary prey of wolves in southeast Alaska (Person 2001). Coupled with the strong territorialism and mostly nonoverlapping home ranges, established healthy populations of coastal wolves may prevent immigrants from penetrating the same locale and have been successfully reproducing, particularly on islands where space is restricted and boundaries are discrete. This situation may not be true for black bears as resistance to immigrants is likely not nearly as intense.

In comparison to other island populations of wolves, POW and KMW approach similar genetic distances from continental wolves as those on Vancouver Island (Roy *et al*. 1994) and exceed genetic distances found for wolves on Banks and Victoria islands of Canada (Carmichael *et al*. 2001). However, these insular wolf populations do not seem to follow a pattern of isolation as drastic as that identified for Kodiak brown bears (Paetkau *et al*. 1998).

Our sampling along the North Pacific coast identified CRD and KEN as distinctive populations (Fig. 2), although this is not supported in analysis of mtDNA (Talbot *et al*. in review). Assignment tests further distinguished the KEN population with the highest proportion of population assignment. The Kenai Peninsula is connected to mainland Alaska by a narrow neck of land and ice which is only 16 km wide, thus providing geographical separation that may support the maintenance of a distinctive peninsular population. Wolves of the Kenai Peninsula and elsewhere in central Alaska are likely the result of colonization from one of the northern refugia (Pedersen 1982). The original populations of wolves on the Kenai Peninsula are assumed to have been extirpated in the early 20th century with the peninsula recolonized from interior Alaska populations in the 1960s (Peterson & Woolington 1982). Our results may support this scenario by indicating a recent bottleneck, but this support is weak and the level of bottlenecking assumed in anecdotal natural history accounts are not supported by our data (see also Talbot *et al*. in review). Pedersen's (1982) review of the taxonomy of modern Kenai wolves, based on morphology, did not distinguish the Kenai wolves from those of interior Alaska. In addition, wolves were repeatedly sighted on the Kenai Peninsula during their supposed extirpation (Peterson *et al*. 1984). Movement into the peninsula is difficult to detect and was not observed during radio telemetry studies conducted between 1976 and 2000 (T. Bailey, personal communication) although emigrating wolves have been observed and fixation indices indicate random mating (Table 1). A potentially unique population could have persisted at low numbers, and after mixing with recent dispersers, resulted in this signal of genetic divergence.

In contrast, the assignment test largely failed to assign CRD to a single cluster (e.g. 23% of mean individual assignment was in the southeast Coastal group). For all other populations, over 90% of individuals were assigned to their respective group. CRD is thought to have become established following the Good Friday Earthquake in 1964. The weak assignment test may indicate that the CRD is a contact zone between Coastal and Continental populations, however, mtDNA data do not show admixture (Talbot *et al*. in review; Weckworth *et al*. unpublished). Observations of wolves on the CRD were apparently limited or absent until recent decades, perhaps because of rapid extermination as reported on KEN (Peterson *et al*. 1984), and a limited ungulate prey base on the CRD. The introduction and subsequent expansion of moose (*Alces alces*) to the CRD during the period 1949–1958 apparently allowed wolves to colonize the area by the early 1970s (Stephenson *et al*. unpublished). Thus, the population of wolves on CRD, like KEN, is considered to have originated from a small number of individuals, presumably from areas to the north via the Copper River during the winter months (Stephenson *et al*. unpublished). Subsequent to the Good Friday Earthquake, vegetation succession in the area and resulting alternative prey availability may have further altered predator/prey relationships on CRD, resulting in increased availability of nonmammalian prey (Stephenson & Van Ballenberghe 1995). However, it is not clear whether this

added prey availability increased wolf density in the area. Stephenson *et al*. (unpublished) suggest that wolves inhabiting the Copper/Bering River delta area represent an essentially closed population, because of natural barriers restricting movement into or out of the area. Dispersing wolves are thought to remain in the area, and despite an apparent lack of vacant territory, no emigrating wolves were detected during radio collar studies conducted from 1992 to 1996 (Carnes *et al*. unpublished). Our microsatellite data, however, are inconsistent with the hypothesis that CRD is an isolated population.

Within southeast coastal Alaska, we originally designated four populations (Fig. 2). The assignment test indicates two distinct clusters in southeast Alaska, POW and all other individuals. Among these, POW is distinctive with pairwise F_{ST} values considerably larger than other southeast pairwise comparisons and a pattern of assignment that may reflect isolation or reduced gene flow from other Coastal populations. This finding is consistent with biogeographical assessments of the archipelago (MacDonald & Cook 1996). Nearshore islands, such as Revillagigedo, and the Mitkof/Kupreanof 'peninsula' tend to show close connectivity with the mainland while the Prince of Wales Island complex is largely isolated within the region. The distinctive POW population corroborates previous studies identifying Prince of Wales Island as a centre of endemism for flying squirrels (*Glaucomys sabrinus*, Bidlack & Cook 2002), deer mice (*Peromyscus keeni*, Lucid & Cook 2004) and ermine (*Mustela erminea*, Fleming & Cook 2002). Overall, the assignment analyses indicate fewer distinctive populations (Table 3) than our originally assigned populations. More extensive sampling throughout the North Pacific coast, particularly in southerly regions, may help clarify population structure of grey wolves.

Geffen *et al*. (2004) suggest that environmental conditions influence dispersal decisions in wolves. Local climate, habitat features, and prey become imprinted on developing grey wolves, and thus young dispersers may seek out familiar landscapes (Geffen *et al*. 2004). Dispersing wolves that select familiar ground have a better chance of survival (Gese & Mech 1991). In northern Canada, behavioural differences may relate to the genetic differentiation of wolves that hunt migrating caribou from other nearby resident wolves that prey on nonmigratory species (Carmichael *et al*. 2001). Habitat, climatic features, and prey base of coastal southeast Alaska differs substantially from inland continental regions east of the Coast Range. These differences likely decrease gene flow and further facilitate differentiation.

Person *et al*. (1996) identified lack of sufficient prey base and over-harvest of wolves as the primary threat to their persistence in southeast Alaska. Salmon runs provide only a seasonal food source, and deer populations are predicted to decline as a result of human mediated changes to habitat (Wallmo & Schoen 1980; Schoen *et al*. 1988). Wolf populations in southeast Alaska likely number 700–1100 individuals, with total annual mortality rates exceeding 35% in some areas (Person *et al*. 1996). Several studies suggest that such rates of mortality for wolves are unsustainable (Gasaway *et al*. 1983; Peterson *et al*. 1984; Fuller 1989). The impacts of increased harvest pressure, decreased prey base, and insular vulnerability, synergistically affected by timber management practices along the North Pacific coast during the last century, are likely to be exacerbated by continued clear-cutting and road construction (Parker *et al*. 1996). These practices have been particularly intense on Prince of Wales Island, where road building and logging have been expansive and harvest rates are estimated at 30%–40%. Further, over 80% of dispersers on POW are killed before reproducing, 70% of this mortality can be attributed to hunting and trapping. Increased access to remote areas has been shown to impact populations through events such as fragmentation or increased anthropogenic interactions (Thurber *et al*. 1994; Mladenoff *et al*. 1999). Areas of higher road density may be biological sinks (Pulliam 1988) that are not sustainable habitat on their own (Mladenoff *et al*. 1997). Similar effects have influenced wolf populations elsewhere (Mladenoff *et al*. 1995), further highlighting the need for continued monitoring of this biologically diverse and complex region (Cook & MacDonald 2001).

Conclusions

The microsatellite data described herein suggest that within the relatively small geographical area of southeast Alaska, coastal wolves have diverged from adjacent continental populations in the Pacific Northwest, have retained fairly high genetic variation, and exhibit greater geographical structuring than continental populations do.

Lack of a fossil record suggests that wolves have only occupied southeast coastal Alaska during the Holocene. The microsatellite data suggest that, subsequent to the Last Glacial Maximum, expansion of wolf populations into southeast Alaska was followed by isolation from surrounding populations. Wolves of southeastern Alaska differ significantly in allele frequencies at nuclear loci, and thus meet at least one of the genetic criteria widely used to identify evolutionary significant units (ESUs) or management units (MUs) (*sensu* Moritz 1994). Additional genetic criteria for identification of unique units of evolution, such as significant differences, or reciprocal monophyly, in genes of the mtDNA, should be investigated for these wolves. In addition, emphasis should be placed on the maintenance of adaptive diversity (Crandall *et al*. 2000), especially when considering evolutionary processes in conservation biology. Certainly, the contemporary demographic independence of the wolves of POW should be considered in any plan used to manage those populations or substantially alter habitat, because insular populations cannot be expected to

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easily recruit from neighbouring mainland populations. Our genetic data, when interpreted in light of past morphological research, are consistent with patterns of variation observed in other mammalian species inhabiting southeastern Alaska, and suggest that coastal wolves (*Canis lupus ligoni*) may represent a previously unrecognized and significant component of diversity in North American wolves.

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Appendix

Frequency of occurrence of alleles for 11 microsatellite loci collected from populations of *Canis lupus*. Population abbreviations as in Table 1

		Populations								
		Coastal				Continental				
Locus	Allele	KMW	REV	POW	MCS	FAI	CRD	KEN	BC	YUK
C030	144	—	—	0.012	—	—	—	—	—	—
	145	—	—	0.012	—	0.034	0.036	0.121	0.121	0.071
	149	0.250	0.271	0.345	0.167	0.052	0.357	0.152	0.414	0.071
	150	—	—	—	—	0.121	0.036	—	0.034	—
	151	0.077	0.125	0.238	0.056	0.052	—	—	0.086	0.143
	152	—	—	—	—	0.034	—	0.015	—	0.036
	154	—	—	—	—	0.017	—	0.045	—	—
	155	—	—	—	—	0.052	0.036	—	—	—
	157	0.096	0.083	0.012	0.222	0.017	—	—	0.069	0.107
	159	—	0.042	—	0.111	0.241	—	0.242	0.017	0.036
	160	—	—	—	—	—	—	—	0.017	—
	161	0.442	0.104	0.071	0.278	—	0.250	0.030	0.034	0.071
	163	0.135	0.333	0.298	0.167	0.259	0.286	0.394	0.155	0.286
	165	—	0.042	0.012	—	—	—	—	0.034	0.179
	167	—	—	—	—	0.121	—	—	0.017	—
C109	142	0.019	—	—	0.111	—	0.143	—	0.017	—
	144	0.288	0.435	0.512	0.333	0.268	0.250	0.258	0.448	0.357
	146	—	—	—	—	0.125	0.143	0.227	—	0.357
	148	—	0.022	—	—	0.554	0.357	0.515	0.276	0.214
	150	0.038	0.022	—	0.056	—	0.036	—	0.190	0.036
	151	—	0.022	—	—	—	—	—	—	—
	152	0.654	0.500	0.488	0.500	0.054	0.071	—	—	0.036
	154	—	—	—	—	—	—	—	0.069	—
C123	139	—	—	0.012	—	—	—	—	—	—
	141	—	—	0.024	—	—	—	—	0.033	—
	145	0.942	0.958	0.845	1.000	0.672	0.929	0.636	0.750	0.536
	147	0.019	0.042	0.119	—	0.155	—	0.242	—	0.214
	149	—	—	—	—	0.086	—	—	0.017	0.143
	151	0.038	—	—	—	0.086	0.071	0.121	0.200	0.107
C172	155	0.788	0.761	0.762	0.500	0.052	0.357	0.530	0.433	0.357
	157	0.212	0.239	0.238	0.500	0.948	0.643	0.470	0.567	0.643
C173	103	—	—	—	—	0.190	0.250	—	0.300	0.250
	105	—	—	—	—	—	—	—	0.033	—
	107	0.769	0.375	0.298	0.444	0.414	0.500	0.712	0.167	0.179
	109	—	0.063	—	0.111	0.121	—	0.152	0.200	0.071
	111	0.212	0.417	0.500	0.444	0.207	0.107	0.136	0.300	0.250
	113	0.019	0.146	0.202	—	0.069	0.143	—	—	0.250
C203	120	0.038	—	—	—	—	—	—	—	—
	122	0.462	0.341	0.598	0.333	0.155	0.107	0.455	0.200	0.346
	126	—	—	—	—	0.034	—	—	—	—
	128	—	—	—	—	—	—	—	0.040	—
	130	0.038	—	0.024	—	0.431	0.179	0.015	0.340	0.500
	132	0.135	0.227	0.183	0.111	0.121	0.500	0.227	0.100	0.077
	134	—	—	—	—	0.103	—	0.061	0.020	—
	136	0.327	0.432	0.195	0.556	0.103	0.214	0.242	0.300	0.077
	140	—	—	—	—	0.034	—	—	—	—
	142	—	—	—	—	0.017	—	—	—	—

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Appendix I *Continued*

		Populations								
		Coastal				Continental				
Locus	Allele	KMW	REV	POW	MCS	FAI	CRD	KEN	BC	YUK
C204	201	0.558	0.500	0.037	0.278	0.121	0.179	0.455	0.050	0.143
	203	—	—	—	—	0.241	—	—	0.100	0.036
	207	0.423	0.500	0.963	0.611	0.483	0.714	0.545	0.317	0.607
	209	0.019	—	—	0.111	0.155	0.107	—	0.533	0.214
C213	141	—	—	—	—	0.017	—	—	—	—
	154	—	—	—	—	—	—	0.045	0.017	—
	156	—	0.083	0.049	—	0.138	—	0.273	0.067	0.107
	157	0.020	0.083	0.012	0.167	—	—	—	0.167	0.036
	158	—	—	—	—	—	—	—	0.017	—
	159	0.060	0.229	0.805	0.111	0.172	0.346	0.091	0.067	—
	160	—	0.042	—	—	—	—	—	0.067	—
	161	0.920	0.521	0.073	0.667	0.259	0.115	0.258	0.267	0.429
	162	—	0.021	0.061	—	0.103	0.115	0.152	0.017	0.071
	163	—	—	—	—	0.069	—	—	0.183	0.071
	164	—	—	—	—	0.190	0.423	0.182	0.100	0.286
	166	—	0.021	—	0.056	0.052	—	—	0.033	—
C225	160	0.481	0.417	0.440	0.667	0.500	0.385	0.833	0.362	0.536
	162	0.308	0.354	0.417	0.222	0.138	0.154	0.167	0.362	0.393
	164	0.212	0.229	0.143	0.111	0.138	0.462	—	0.224	0.071
	166	—	—	—	—	0.224	—	—	0.052	—
C250	134	0.019	—	—	—	0.121	—	—	—	0.071
	136	0.038	0.021	0.012	0.056	0.086	—	0.303	0.241	—
	138	0.038	0.042	0.071	—	0.017	0.308	—	0.172	0.036
	140	0.788	0.646	0.595	0.333	0.569	0.692	0.424	0.276	0.643
	142	0.096	0.083	0.286	0.222	0.086	—	—	0.017	0.036
	144	0.019	0.208	0.036	0.389	0.121	—	0.273	0.190	0.179
	148	—	—	—	—	—	—	—	0.103	0.036
C377	147	—	0.063	0.134	0.056	0.034	0.179	0.030	0.067	0.036
	149	0.096	0.146	0.024	0.111	0.121	0.036	—	0.067	0.107
	157	0.173	0.125	—	0.167	—	—	—	0.117	0.036
	159	—	—	—	—	0.052	—	—	0.033	0.036
	161	—	—	—	—	—	—	—	0.033	—
	163	0.346	0.292	0.598	0.333	0.259	0.250	0.106	0.133	0.071
	165	—	—	—	—	—	—	—	0.083	—
	167	0.385	0.375	0.244	0.333	0.534	0.536	0.864	0.467	0.714

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