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Molecular Phylogeny and SNP Variation of Polar Bears (*Ursus maritimus*), Brown Bears (*U. arctos*), and Black Bears (*U. americanus*) Derived from Genome Sequences

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Abstract

We assessed the relationships of polar bears (*Ursus maritimus*), brown bears (*U. arctos*), and black bears (*U. americanus*) with high throughput genomic sequencing data with an average coverage of 25× for each species. A total of 1.4 billion 100-bp paired-end reads were assembled using the polar bear and annotated giant panda (*Ailuropoda melanoleuca*) genome sequences as references. We identified 13.8 million single nucleotide polymorphisms (SNP) in the 3 species aligned to the polar bear genome. These data indicate that polar bears and brown bears share more SNP with each other than either does with black bears. Concatenation and coalescence-based analysis of consensus sequences of approximately 1 million base pairs of ultra-conserved elements in the nuclear genome resulted in a phylogeny with black bears as the sister group to brown and polar bears, and all brown bears are in a separate clade from polar bears. Genotypes for 162 SNP loci of 336 bears from Alaska and Montana showed that the species are genetically differentiated and there is geographic population structure of brown and black bears but not polar bears.

Key words: *phylogeny, polar, brown, black bears, Ursus, ultraconserved elements, molecular clock, single nucleotide polymorphism*

Phylogenetic assessment of DNA sequences for individual loci may not reflect the true relationships of taxa (Pamilo and Nei 1988; Heled and Drummond 2010). For example, discordance of mitochondrial DNA (mtDNA) and nuclear DNA phylogenies of animals has been reported in such diverse taxa as *Drosophila* (Powell 1983; DeSalle and Giddings 1986), reptiles (Wiens et al. 2010), and large mammals including elephants (*Loxodonta*; Roca et al. 2005), deer (*Odocoileus*; Carr et al. 1986), and bears (*Ursus*; Cronin et al. 1991). The case of bears is particularly interesting because there have been several recent and variable estimates of molecular phylogeny and divergence times of brown bears (*Ursus arctos*), polar bears (*U. maritimus*), and American black bears (*U. americanus*; Lindqvist et al. 2010; Davison et al. 2011; Edwards et al. 2011; Hailer et al. 2012, 2013; Miller et al. 2012; Cahill et al. 2013; Nakagome et al. 2013). These studies describe a nuclear phylogeny with brown and polar bears in separate lineages, but a paraphyletic mtDNA phylogeny in which polar bears and brown bears from Admiralty, Baranof, and Chichagof islands (ABC) in southeast Alaska are in a clade separate from other brown bears. In addition, polar bears and ABC brown bears (but not other brown bears) have shared

common ancestry of up to 10% of the nuclear genome (Hailer et al. 2012; Miller et al. 2012) including 6.5% of the X chromosome loci (Cahill et al. 2013). This is hypothesized to have resulted from interspecies hybridization when brown bears replaced polar bears during postglacial colonization on the ABC islands (Cahill et al. 2013). However, extant populations of polar bears and brown bears have separate gene pools with little or no hybridization (Cronin and MacNeil 2012; Hailer et al. 2012; Cronin et al. 2013).

The fossil record suggests the brown bear/polar bear lineage diverged from the black bear lineage roughly 2 million years ago (Kurtén 1968; Wayne et al. 1991), whereas molecular clock divergence time estimates from proteins, mtDNA, and nuclear DNA sequences vary widely and suggest these lineages split from 0.6 to 6.7 Ma (Goldman et al. 1989; Waits et al. 1999; Yu et al. 2004, 2007; Lindqvist et al. 2010; Hailer et al. 2012; Miller et al. 2012). Polar bears and brown bears are thought to have evolved from a common ancestor during the Pleistocene (Kurtén 1964, 1968), and a polar bear fossil from the last interglacial (Eemian) period 110 000–130 000 years ago established this as their minimum time of divergence (Ingólfsson and Wiig 2008; Alexanderson et al. 2013). Molecular clock estimates of the divergence time of polar bears and brown bears vary widely depending on the genetic markers used. These include divergences of 2–3 Ma with proteins (Goldman et al. 1989), 0.11–1.7 Ma with mtDNA (Talbot and Shields 1996a; Yu et al. 2004, 2007; Arnason et al. 2007; Bon et al. 2008; Krause et al. 2008; Lindqvist et al. 2010; Davison et al. 2011; Edwards et al. 2011), and 0.34–2.0 Ma with nuclear DNA sequences (Yu et al. 2004; Edwards et al. 2011; Hailer et al. 2012). An analysis of genome sequences estimated the polar bear–brown bear divergence at 4–5 Ma with subsequent periods of gene flow between lineages (Miller et al. 2012).

In this paper, we reassess the phylogeny of polar bears, brown bears, and black bears using genetic markers not previously used on bears: ultraconserved elements (UCE) derived from genome sequences. UCE are highly conserved short DNA sequences that are shared by different organisms and are particularly useful for phylogeny estimation from genome sequence data (Faircloth et al. 2012). UCE have been useful in resolving difficult evolutionary relationships (e.g., birds, McCormack et al. 2013; turtles, Crawford et al. 2012). UCE can provide a useful measure of phylogeny in bears because they are numerous in the nuclear genome (Stephen et al. 2008), easily identifiable, the flanking regions show increasing amounts of sequence variability with increasing distance from the UCE, are generally independent loci (Faircloth et al. 2012), and are free of paralogy and retroelement insertions in most cases (Derti et al. 2006; Simons et al. 2006). Genetic variation was also assessed within and between polar, brown, and black bears from North America with single nucleotide polymorphisms (SNP) derived from genome sequences. Our objectives were to compare interspecies DNA sequence divergence to estimate the phylogeny of 3 bear species and quantify inter- and intraspecies SNP variation.

Materials and Methods

Genome Sequencing and Identification of Variation

Two genome mapping approaches were utilized. One approach used the polar bear genome (Li et al. 2011) as reference to determine sequence variation including single nucleotide variants (SNV), SNP, indels, and UCE among bear species on a whole genome scale. The second approach used the annotated giant panda (*Ailuropoda melanoleuca*) genome (Li et al. 2010) as reference to select high quality SNP in target genes and intergenic regions to genotype a large number of samples for population genetic analyses.

DNA from 2 to 4 individuals of each species and geographic location were combined to make 7 DNA pools, including 2 pools of polar bears, 2 pools of black bears, 1 pool of ABC brown bears, and 2 pools of other brown bears (non-ABC brown bears, Figure 1 and Table 1). DNA libraries for each pool were made from 6 μg genomic DNA using the Illumina Genomic DNA sample kit (Illumina Inc., San Diego, CA). Each bear species' (polar, brown, and black) genome was sequenced at an average of 25× coverage using the Illumina HiSeq platform. A total of 1.4 billion 100 base-pair (bp) paired-end (PE) reads were mapped to the polar bear genome and the panda genome as references. Illumina 100-bp PE reads were assembled and analyzed in CLC Genomics Workbench 5.5.1 (CLC Bio, Aarhus, Denmark).

Numbers of SNV, SNP, and indels were quantified within and between species using the genome sequences from the 7 DNA pools aligned with the polar bear genome. SNV include sites with different nucleotides or indels and SNP include sites with different nucleotides (i.e., SNV = SNP + indels). We calculated the number of SNP that are shared by each pair of species and by all 3 species. The number of SNP that are not shared by each pair of species was calculated by subtracting the number of SNP shared by each pair of species plus the number shared by all 3 species from the total number of SNP.

Identification of UCE

UCE and flanking regions were used for phylogeny inference. To identify UCE in bears and other Carnivora, the 5561 UCE probes (each 120 bp) from Faircloth et al. (2012) were blasted (expected value = 1E-15) against the polar bear (Li et al. 2011), giant panda (Ensembl 66), domestic dog (*Canis familiaris*; Ensembl 66), house cat (*Felis catus*; Ensembl 66), and polecat (*Mustela putorius*; Ensembl 66) genomes using GeneiousR6 (2013). All single hit UCE blast hits were retained from each genome and extended 250 bp on both the 5' and 3' flanking ends using GeneiousR6. This resulted in the identification of 4114 homologous UCE sequences (each of which was approximately 620 bp in length) in the genome sequences of each of the 5 species.

Four of our *Ursus* sequence data sets were then mapped to the polar bear genome using GeneiousR6 default settings: combined pools 1 and 2 (polar bears, Table 1); pool 3 (ABC brown bears); combined pools 4 and 5 (non-ABC brown bears); and combined pools 6 and 7 (black bears).

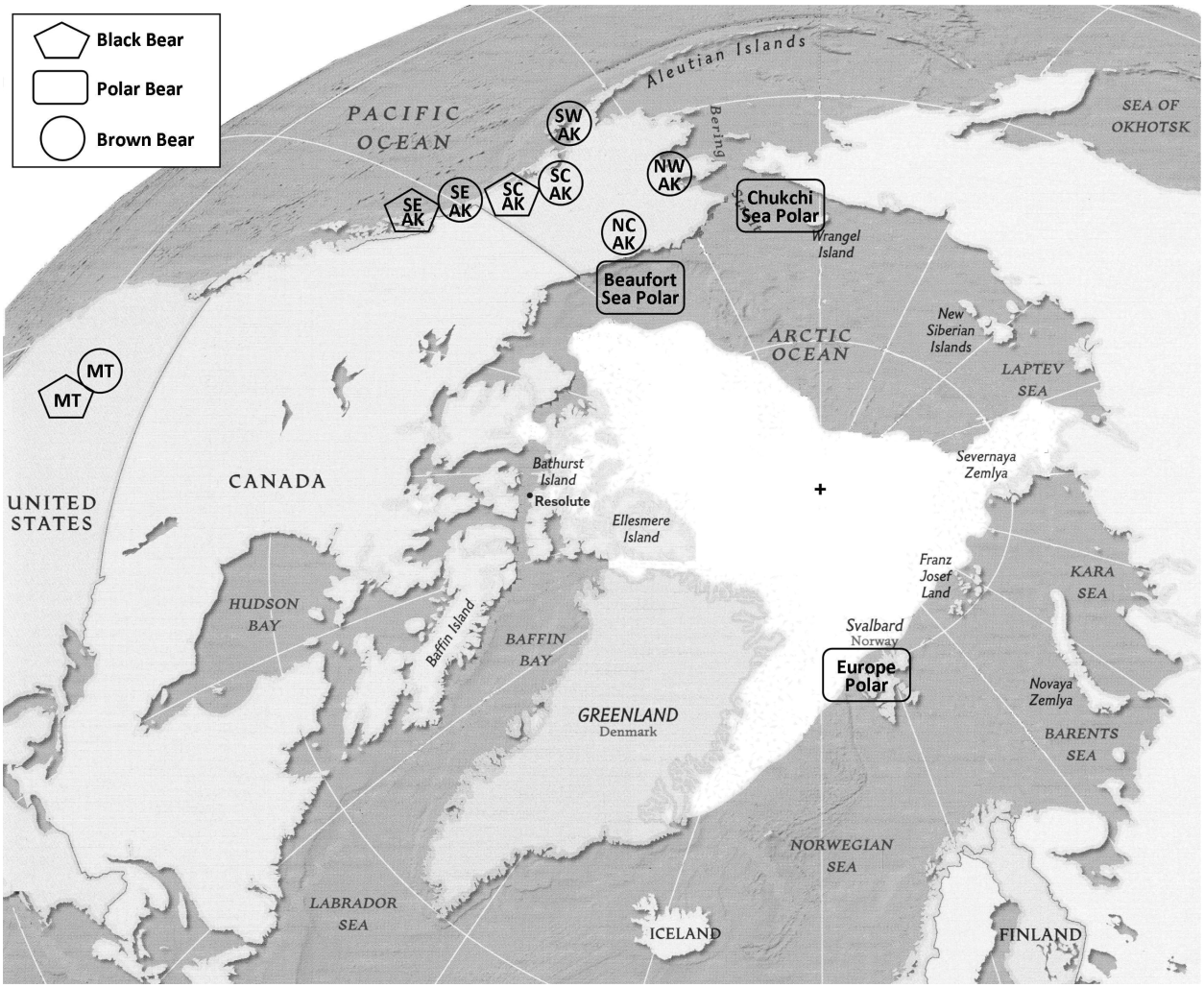

Figure 1. Map of bear sampling locations. AK, Alaska; MT, Montana; NC, northcentral; NW, northwest; SC, southcentral; SE, southeast; SW, southwest.

A consensus sequence corresponding to each 620-bp UCE region with coverage greater than 5× was created for each of the 4 *Ursus* data sets. The consensus sequences included variable nucleotides at individual positions within each of the *Ursus* data sets (i.e., SNP that varied within or between individual animals in the pooled sequences). All homologous UCE regions from the 4 Ensembl genomes (panda, dog, cat, and polecat) were aligned with our 4 *Ursus* UCE data sets (8 taxa total) using Muscle (Edgar 2004) default settings. The aligned sequences were then processed with trimAl (Capella-Gutierrez et al. 2009) to remove all alignment columns with gaps in more than 12.5% of the sequences and similarity scores lower than 0.001. These values were chosen to identify and remove all alignment regions that were either unsequenced in one or more taxa or ambiguously aligned. For subsequent analyses, we retained 1681 UCE region alignments that contained all 8 taxa with no more than 2 ambiguous base pairs (i.e., a SNP within a taxon or low-quality nucleotide calls). Models of molecular evolution for each segment were estimated using default settings in Molecular Evolutionary Genetics Analysis Computing Core (MEGA-CC) (Kumar et al. 2012).

Phylogenetic Analyses

We estimated phylogeny with concatenation and coalescence analyses of UCE sequences. For the first analysis, we conducted maximum likelihood concatenation analysis with RAxML7.4.2 (Stamatakis 2006). The 1681 UCE region alignments were concatenated into a 13-partition alignment (996 381 bp). Each partition consisted of all UCE region alignments with the same estimated model of molecular evolution as determined by MEGA-CC (Supplementary Table 1). RAxML7.4.2 implements only one of the models tested by MEGA-CC (GTR [general time reversible] model), so we implemented the most complex model suggested by MEGA-CC (GTR + Γ + I) for each partition of the RAxML analysis. The RAxML analysis included 500 replicates, started from randomized MP starting trees, and used the fast hill-climbing algorithm with all other free parameters estimated.

Table 1 Bear samples used in pools for genome sequencing

Sample number/geographic location	Sex
Pool 1: Female POLAR BEARS from Beaufort and Chukchi Seas, Alaska	
2899/Barrow, Alaska	Female
3003/Point Lay, Alaska	Female
3069/Gambell, St Lawrence Island, Alaska	Female
2848/Point Hope, Alaska	Female
Pool 2: Male POLAR BEARS from Beaufort and Chukchi Seas Alaska	
2951/Point Hope, Alaska	Male
3061/Gambell, St Lawrence Island, Alaska	Male
3063/Diomede Island, Alaska	Male
Pool 3: BROWN BEARS from Southeast Alaska ABC islands	
5/Chichigoff Island, Southeast Alaska	Male
7/Admiralty Island, Southeast Alaska	Male
10/Chichigoff Island, Southeast Alaska	Male
40/Admiralty Island, Southeast Alaska	Male
Pool 4: BROWN BEARS from Alaska non-ABC islands	
37/Southeast Alaska Mainland	Male
41/Southeast Alaska Mainland	Male
Pool 5: BROWN BEARS from Alaska non-ABC	
45/Bunko Creek, South Central Alaska	Male
4/Unit 23, Northwest Alaska	Male
Pool 6: BLACK BEARS from Alaska	
46/Yetna River, Southcentral Alaska	Female
3/Southeast Alaska Unit 1 mainland	Male
Pool 7: BLACK BEARS from Alaska	
1/Southeast Alaska Unit 1 mainland	Male
42/Blackstone Bay, Prince William Sound, Alaska	Female
44/East Chenega Island, Prince William Sound, Alaska	Unknown
47/Southcentral Alaska, Byers Lake	Female

The second analysis was the coalescence-based model in Maximum Pseudo-liklihood Estimate of the Species Tree (MP-EST) (Liu et al. 2010) and was used to account for possible differences between gene trees (i.e., a tree for each UCE region) and the species tree. MP-EST utilizes a maximum pseudo-likelihood approach to estimate the species trees with branch lengths in coalescent units. We used RAxML7.4.2 to estimate the phylogeny for each of the 1681 UCE region alignments. MEGA-CC was used to determine whether or not to include a gamma distribution or invariant sites. The domestic cat was designated as the outgroup. All other RAxML7.4.2 settings were the same as in the concatenation analysis. The MP-EST analysis started from a random tree and all other settings were default values.

We also calculated the number of substitutions and substitutions/site (p-distance) between the UCE sequences for each pair of taxa. The p-distance is the number of substitutions between 2 taxa divided by the total number of nucleotides (996381).

SNP Selection, Genotyping, and Population Genetic Analysis

To assess SNP variation within and between species for large numbers of bears, 180 SNP (120 SNP in intergenic regions and 60 SNP in coding regions) were selected from the SNP discovered in the genomic sequences of the 7 bear DNA pools aligned against the annotated panda genome. These SNP were selected from the 20 largest panda scaffolds (>5 million bp) placing each SNP 1 million nucleotides apart and included SNP with sequence read frequencies (i.e., the number of reads corresponding to each SNP variant nucleotide) of 90/10, 80/20, 70/30, 60/40, and 50/50. For example, if a SNP is G/T, then for the first case 90% of the reads will be G and 10% will be T. All 3 species are represented in the selected SNP because we used all 7 DNA pools for SNP selection. Details on the selected SNP are in Supplementary Table 2, and sequences for primers for the 180 SNP were submitted to dbSNP at NCBI (Supplementary Table 3).

Three hundred and eighty-four bears were genotyped for the 180 SNP including black bears from 2 geographic regions in Alaska and from Montana, brown bears from 5 regions in Alaska and from Montana, and polar bears from 2 regions in Alaska and 1 polar bear from Svalbard, Europe (Figure 1 and Table 2). Replicate samples, samples with genotypes at <84% of the loci, and samples with uncertain species or geographic identity were deleted from further analyses. Genotypes were determined with the Sequenom MassARRAY(R) platform (GeneSeek Inc., Lincoln, NE) and statistics were calculated with SNP variation suite (SVS) version 7 (Golden Helix Inc., Bozeman, MT). Loci that did not produce reliable diploid genotypes were deleted from the analysis. Tests for linkage disequilibrium (LD) between pairs of SNP loci across all populations were done in GENEPOP version 4.1 (Raymond and Rousset 1995) considering a significance level of $\alpha = 0.05$ and sequential Bonferroni correction (Holm 1979; Gaetano 2013). One of each pair of loci with significant LD was excluded from the analysis.

Hardy–Weinberg equilibrium, observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated for the SNP loci with GenALEx 6.5 (Peakall and Smouse 2012). Deviation from Hardy–Weinberg genotype proportions was tested with $\alpha = 0.05$ and sequential Bonferroni correction. Differentiation of black bears, polar bears, ABC brown bears, and non-ABC brown bears was assessed with the numbers of alleles restricted to 1 species or population (i.e., private alleles) and shared by >1 species. Differentiation of species and geographic regions within species was also assessed with Principal Coordinate Analysis (PCoA; Orloci 1978) of pairwise individual genetic distances (Smouse and Peakall 1999) in GenALEx.

Results

Genome Sequencing and Identification of Variation

A total of 1.4 billion PE 100-bp reads were obtained by sequencing the 7 DNA pools. After quality control analysis, 6% of the sequence reads did not pass standard quality filters and were discarded from further analysis. The total number of reads and statistics on the reference assembly mapping are shown in Table 3. Note that black bears map 6% more sequences to the polar bear genome than do brown bears.

Table 2 Sampling locations, observed (H_o) and expected (H_e) heterozygosity, and Hardy–Weinberg results in bear species and regions for 162 SNP genotypes

Species	Geographic region	N	162 loci				50 coding loci			112 intergenic loci		
			H_o	H_e	No. (%) P[a]	HW[b]	H_o	H_e	No. (%) P[a]	H_o	H_e	No. (%) P[a]
Black	Montana	18	0.0157	0.0199	18 (11)	4	0.0140	0.0195	4 (8)	0.0164	0.0200	14 (13)
Black	Southcentral Alaska	48	0.0153	0.0143	20 (12)	2	0.0092	0.0096	5 (10)	0.0181	0.0164	15 (13)
Black	Southeast Alaska	20	0.0148	0.0205	16 (10)	1	0.0185	0.0272	6 (12)	0.0131	0.0176	10 (9)
Brown	Southcentral Alaska	22	0.1718	0.1790	101 (62)	15	0.1466	0.1552	28 (56)	0.1831	0.1896	73 (65)
Brown	Southeast Alaska[c]	15	0.1692	0.1938	99 (61)	0	0.1137	0.1428	27 (54)	0.1940	0.2166	72 (64)
Brown	Southwest Alaska[d]	3	0.1173	0.1423	59 (36)	0	0.0933	0.1311	18 (36)	0.1280	0.1473	41 (37)
Brown	Northwest Alaska	42	0.1804	0.1811	99 (61)	3	0.1934	0.1907	33 (66)	0.1746	0.1767	66 (59)
Brown	Northcentral Alaska	35	0.1946	0.1895	100 (62)	2	0.1773	0.1692	30 (60)	0.2023	0.1985	70 (63)
Brown	Montana	6	0.1204	0.1320	65 (40)	0	0.0913	0.0856	14 (28)	0.1333	0.1527	51 (46)
Polar	Beaufort Sea	41	0.0535	0.0507	26 (16)	0	0.0615	0.0617	8 (16)	0.0500	0.0458	18 (16)
Polar	Chukchi Sea	85	0.0514	0.0510	29 (18)	0	0.0678	0.0659	9 (18)	0.0441	0.0444	20 (18)
Polar	Europe Svalbard[e]	1	0.0556	0.0278	9 (6)	0	0.0600	0.0300	3 (6)	0.0536	0.0268	6 (5)
Total		336										

[a] Number and % polymorphic loci.

[b] Number of loci not in Hardy–Weinberg genotype proportions.

[c] Includes 12 bears from ABC Islands and 2 bears from the mainland.

[d] Includes 1 bear from Kodiak Island.

[e] 159 loci for this sample.

This is based only on sequence similarity using the 100-bp reads and 90% similarity parameters and the unmapped reads may represent structural differences between species' genomes. Only a small percentage of the total polar bear reads (7.3%) did not match the existing polar bear genome assembly and may represent genome regions not included in the existing bear assembly.

A total of 16 695 686 SNV, including 13 787 055 SNP and 2 908 631 indels were detected in the 3 bear species (Table 3). A total of 9 795 457 SNP are polymorphic SNP (heterozygous in ≥1 species) and 3 991 598 are monomorphic SNP (homozygous with different alleles in different species). The greatest number of shared SNP is between brown bears and polar bears (1 154 159), followed by brown bears and black bears (367 921), and black bears and polar bears (81 548), and there are 12–14 million SNP not shared by the species (Table 3 and Figure 2). This indicates that brown and polar bears share more SNP across the genome than either does with black bears.

Phylogeny Estimation

The 2 phylogenetic analyses using 996 381 bp in 1681 UCE regions resulted in the same topology, shown as a MP-EST coalescence tree (Figure 3) and a RAxML concatenation tree (Supplementary Figure 1). All nodes in the best RAxML tree received 100% bootstrap support. Within Caniformia, Ursidae (*Ursus* and panda) was recovered as monophyletic, with *Mustela* and *Canis* as successive sister groups. Within *Ursus*, black bears were recovered as the sister group to brown and polar bears, and brown bears (ABC and non-ABC brown bears) form a monophyletic group to the exclusion of polar bears. The number of substitutions in the 996 381-bp UCE sequences and substitutions/site p-distance between each pair of taxa reflect these relationships. The p-distances are largest between *Felis*, *Canis*, *Mustela*, and the Ursidae, intermediate within *Ursus*, and smallest between the ABC and non-ABC brown bears (Table 4).

SNP Selection, Genotyping, and Population Genetic Analysis

Of the 180 SNP analyzed, 171 gave reliable genotypes in the 3 species. Nine loci had unreliable genotypes in one or more species and were deleted from further analysis (Supplementary Table 2). This included 2 coding and 4 intergenic autosomal loci and 3 sex chromosome loci. The sex chromosome loci include one (SRY, GL193852.1_141820) that did not result in genotypes for polar bears, one (GL192414.1_2042479) with a rare and a common allele but all genotypes were homozygous, and one (GL192414.1_2044481) that was heterozygous in 95% of the male and only 6% of the female brown and polar bears, and may be homologous loci on the X and Y chromosomes. Of the 171 loci with informative genotypes, 9 pairs of loci of 9441 pairwise locus tests had significant LD including 5 coding and 4 intergenic loci (Supplementary Table 2). One of each pair of these loci was deleted leaving 162 loci (50 coding and 112 intergenic) in the population genetic analysis.

Replicate genotypes were obtained for 16 bears, including 14 bears with 2 replicate genotypes and 2 bears with 3 replicate genotypes for each of the 162 loci (total 5508 replicate genotypes). There were 14 cases (0.003 of the replicates) in which a genotype was not determined in one of the

Table 3 Genome information for DNA sequences obtained from black, brown, and polar bears

	Polar bear	Brown bear	Black bear
A. Reads mapped to polar bear reference genome of 2 308 415 131 bases.			
Mapped reads	378 798 077 (92.6%)	402 836 175 (83.37%)	372 891 512 (89.48%)
Not mapped reads	29 998 993 (7.34%)	80 336 689 (16.63%)	43 827 702 (10.52%)
Reads in pairs	345 432 464 (84.5%)	358 487 710 (74.19%)	324 242 330 (77.81%)
Total reads	408 797 070	483 172 864	416 719 214

	SNV[a]	Total SNP[a]	Polymorpic SNP[b]	SNV coding regions	Nonsynonymous aa change	Synonymous	Indels	Monomorphic SNP[b]
B. SNV and SNP variation in 3 bear species.								
Polar bears	2 618 533	1 962 384	1 615 441	670 959	17 615	8823	656 149	346 943
Black bears	1 059 704	951 931	305 028	316 958	12 868	5838	107 773	646 903
Brown bears	13 017 449	10 872 740	7 874 988	3 552 859	85 454	39 775	2 144 709	2 997 752
Total	16 695 686	13 787 055	9 795 457	4 540 776	115 937	54 436	2 908 631	3 991 598

SNP shared by	Shared	Not shared[c]
C. SNP shared and not shared by 3 bear species.		
Black and brown only	367 921	13 346 139
Black and polar only	81 548	13 632 512
Brown and polar only	1 154 159	12 559 901
		Average

[a] Total SNP = polymorphic SNP + monomorphic SNP; SNV = total SNP plus indels.
[b] Polymorphic SNP = variable within ≥1 species; monomorphic SNP = not variable within a species but different base in different species.
[c] Number of SNP not shared = total SNP (13 787 055) minus (SNP shared by 2 species + SNP shared by all 3 species (72 995)).

replicates (9 of these were at locus GL192347.1_2313568). Forty-six of the replicate genotypes (0.008) were different for the same bear, indicating a genotyping error rate of <1%.

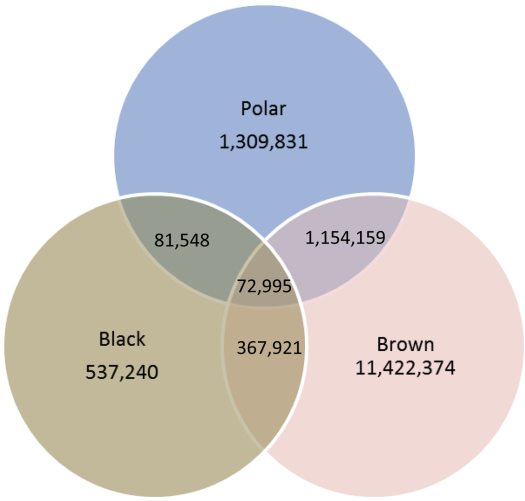

Figure 2. SNP distribution in polar bears, brown bears, and black bears.

This includes 44 cases (0.008) in which 1 replicate had a heterozygous genotype including the allele that was homozygous in the other replicate, and 2 cases (0.0004) in which each replicate was homozygous for a different allele. Replicate sample genotypes (N = 18), samples with genotypes at <84% of the loci (N = 25), and samples with uncertain geographic or species identity (N = 5) were deleted leaving 336 bears in the analysis, including 123 brown bears in 6 geographic regions, 127 polar bears in 3 regions, and 86 black bears in 3 regions.

The proportion of polymorphic loci and heterozygosity in the 162 SNP (including the subsets of 50 coding loci and 112 intergenic loci) is greater in brown bears than in black or polar bears (Table 2). Tests of Hardy–Weinberg genotype proportions in each species/geographic region resulted in 27 of 699 comparisons that were significantly different from expected. This included 1–4 loci in the 3 black bear populations, 0 loci in the polar bear populations, and 2–15 loci in the Alaska brown bear populations (Table 2). These results are not definitive because of small sample sizes for some populations and potential subpopulation structure within the large geographic regions sampled.

The distribution of alleles for the 162 SNP loci includes 79 of 324 total alleles (0.24) that are not shared by the species (i.e., private alleles). This includes 29 (0.09) in polar bears

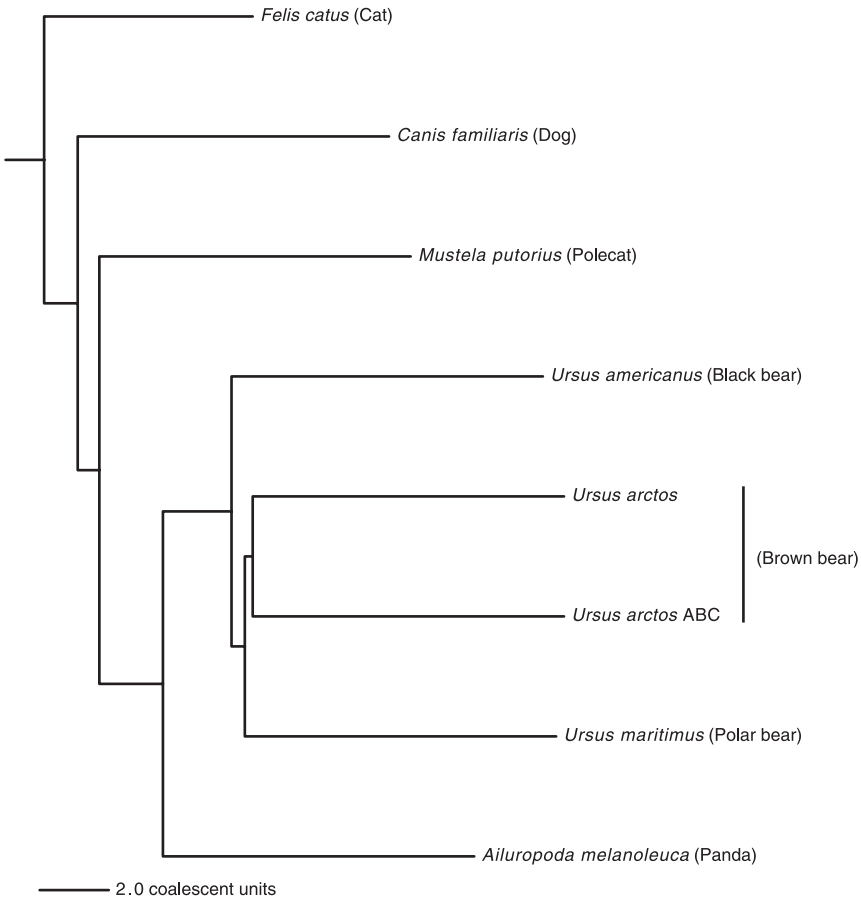

Figure 3. MP-EST coalescence-based tree reconstructions for the 1681 UCE regions alignments.

Table 4 a. Number of substitutions (below diagonal) and substitutions/site (above diagonal) of 996 381 UCE nucleotides between carnivore taxa b. Average substitutions/site among carnivore taxa

	Panda	Dog	Cat	Mustelid	Black bear	Brown bear	ABC brown bear	Polar bear
a. Panda		0.0337	0.0411	0.0343	0.0122	0.0194	0.0119	0.0119
Dog	33 594		0.0422	0.0396	0.0330	0.0327	0.0326	0.0326
Cat	40 982	42 072		0.0467	0.0404	0.0402	0.0411	0.0401
Mustelid	34 145	39 407	46 544		0.0336	0.0333	0.0332	0.0332
Black bear	12 169	32 857	40 283	33 467		0.0024	0.0023	0.0023
Brown bear	11 896	32 544	40 019	33 161	2362		0.0008	0.0012
ABC brown bear	11 865	32 511	39 966	33 127	2323	810		0.0012
Polar bear	11 873	32 516	39 992	33 129	2323	1166	1156	

Taxa compared	Average substitutions/site
b. Average substitutions/site among carnivore taxa	
Cat vs. (dog, mustelid, panda, bears)	0.04170161
Dog vs. (mustelid, panda, bears)	0.03402798
Mustelid vs. (panda, bears)	0.03352713
Panda vs. bears	0.01385737
Black bear vs. (brown/polar bear)	0.00234448
ABC brown vs. polar bear	0.00116020
Non ABC brown vs. polar bear	0.00117024
ABC vs. non-ABC brown bear	0.00081294

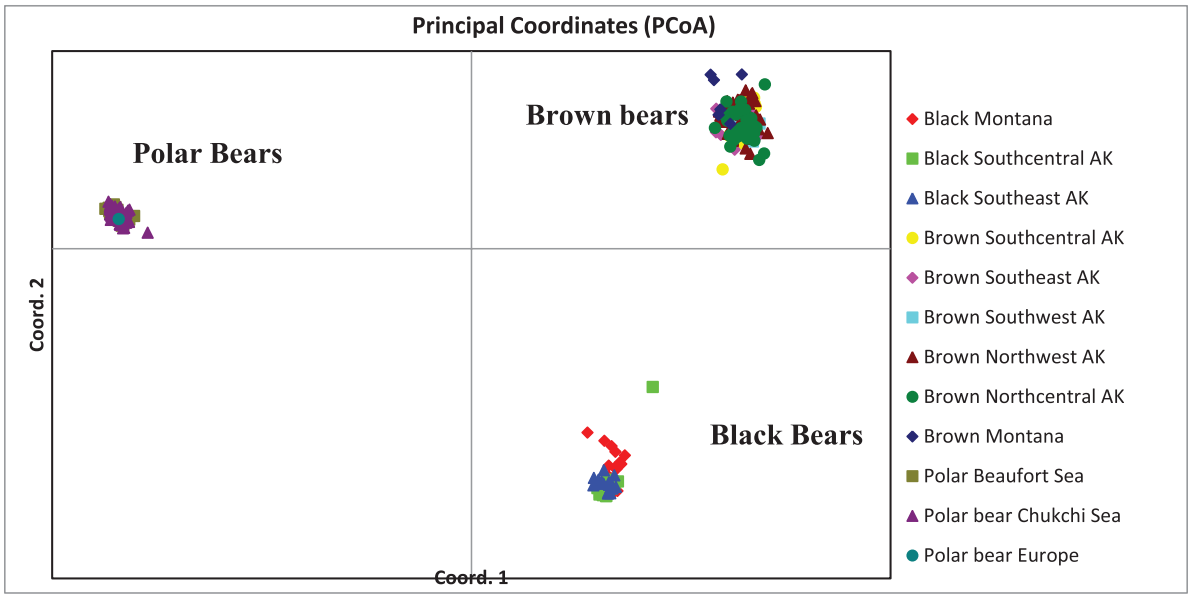

Figure 4. Relationships of 3 bear species and populations from different geographic regions from PCoA analysis of individual animal genetic distances for 162 SNP loci. ABC brown bears include 13 of the 15 bears in the brown southeast AK samples.

only, 50 (0.15) in brown bears only, and 0 in black bears only. Nineteen of the 29 private alleles in polar bears are fixed (i.e., frequency = 1.0), whereas only 2 of the 50 private alleles in brown bears are fixed. Alleles shared by 2 of the 3 species include 49 (0.15) alleles shared by brown bears and polar bears, 79 (0.24) alleles shared by black bears and brown bears, 6 (0.02) alleles shared by black bears and polar bears, and 111 alleles (0.34) are shared by all 3 species. These proportions for all 162 loci are similar to those for coding and intergenic loci (Supplementary Table 4). Of the 49 alleles shared by brown and polar bears, 18 are shared by polar, ABC, and non-ABC brown bears, 29 are shared by polar bears and non-ABC brown bears but not with ABC brown bears, and 2 are shared by polar bears and ABC brown bears but not with non-ABC brown bears. These results are preliminary because of the relatively small number of ABC brown bears sampled (Table 2).

PCoA considering genetic distances between individuals was also used to describe the interspecies and interpopulation relationships among the 336 bears for the 162 SNP

loci (Supplementary Table 5). The first 2 coordinate axes explained 95.3% of the variation of the genetic distances and grouped the samples into species, including clustering the ABC and non-ABC brown bears together (Figure 4). The same basic topologies were obtained in PCoA analyses for the subsets of 50 coding loci and 112 intergenic loci as for the 162 loci. The intraspecies genetic distances between the geographic regions are relatively small and explained <5% of the variation in the PCoA analysis. PCoA analysis of each species individually shows brown bears from southeast Alaska (including ABC bears) and Montana cluster separately from brown bears from the other Alaska regions, polar bears are homogeneous among 3 geographic regions, and black bears from southeast Alaska, Montana, and southcentral Alaska cluster separately with some overlap (Supplementary Figure 2).

Discussion

Our genome sequence analysis (Figure 2) complements the bear genome sequences reported by Miller et al. (2012), as both analyses found more SNP shared by polar and brown bears than by these species and black bears. The phylogeny based on UCE is also consistent with other nuclear DNA phylogenies showing black bears as a sister group to the polar/brown bear lineage, and all brown bears (ABC and non-ABC brown bears) in a clade separate from polar bears (Yu et al. 2004; Fulton and Strobeck 2006; Pages et al. 2008; Hailer et al. 2012; Miller et al. 2012). The nuclear phylogenies are in contrast to a paraphyletic mtDNA phylogeny, in which all ABC brown bears sampled to date have mtDNA that occurs in a clade with polar bear mtDNA. Also, up to 10% of the nuclear genome of ABC brown bears has shared common ancestry with polar bears (Hailer et al. 2012; Miller et al. 2012). These patterns have been used to infer evolutionary processes, including lineage sorting of ancestral mtDNA and nuclear polymorphisms and introgressive hybridization (Cronin et al. 1991; Talbot and Shields 1996a, 1996b; Hailer et al. 2012, 2013; Miller et al. 2012; Nakagome et al. 2013). A detailed analysis showed that polar bear mtDNA was likely introgressed into ABC brown bears (Cahill et al. 2013). In this study, it was demonstrated that <1% of the autosomal genome and 6.5% of the X chromosome loci of ABC brown bears are derived from the polar bear genome. MtDNA is predominantly maternally inherited, and X chromosome loci are diploid in females and haploid in males, so the pattern of genes with common ancestry shared by polar and ABC brown bears (i.e., mtDNA > X chromosome > autosomes) is consistent with introgressive hybridization involving the mating of male brown bears and female polar bears. This is hypothesized to have occurred during postglacial replacement of polar bears by brown bears on the ABC islands, perhaps 12 000 years ago (Cahill et al. 2013). Our data cannot contribute to inference about these past processes because our X chromosome SNP loci were not informative and fewer autosomal SNP alleles were shared by ABC brown bears and polar bears than by non-ABC brown bears and polar bears.

Molecular dating of divergence times of bears has been problematic because of uncertainty of the accuracy of fossil calibrations (Cahill et al. 2013). However, we can infer relative divergence times with the average mutation rate of the human genome of 1×10^{-9} substitutions/site/year (Nachman and Crowell 2000) previously applied to bear genomic sequences (Miller et al. 2012; Cahill et al. 2013). Considering this mutation rate and the estimates of UCE substitutions/site (Table 4), the UCE sequences suggest divergence times of polar bear–brown bear 1.2 Ma, black bear–polar/brown bear 2.3 Ma, panda–black/brown/polar bear 13.9 Ma, mustelid–panda/bears 33.5 Ma, dog–mustelid/panda/bear 34.0 Ma, and cat–Caniformia 41.7 Ma (Supplementary Table 6). We acknowledge that the mutation rate for UCE in bears may not be the same as the rate for the human genome, so these divergence times should not be considered absolute values. However, the UCE estimates provide relative divergence times that are consistent with others that applied the human mutation rate to genomic DNA sequences for brown bear–polar bear (1.2 Ma), black bear–brown bear (2.0 Ma), and panda–black/brown/polar bear (12.0 Ma; Cahill et al. 2013).

The UCE divergence time estimate for brown and polar bears is also within the range of estimates from nuclear DNA sequences (0.34–2.0 Ma; Yu et al. 2004; Edwards et al. 2011; Hailer et al. 2012) and the complete mtDNA sequence (0.93–1.71 Ma; Yu et al. 2007). Other mtDNA divergence time estimates for brown and polar bears are more recent (0.11–0.88 Ma; Talbot and Shields 1996a; Arnason et al. 2007; Bon et al. 2008; Krause et al. 2008; Lindqvist et al. 2010; Davison et al. 2011; Edwards et al. 2011) and an estimate from genome sequences (4–5 Ma; Miller et al. 2012) is considerably older than the UCE estimate. The UCE divergence time estimate of the black bear and brown/polar bear lineages is within the range of some other molecular clock estimates for these species (2.0–3.5 Ma, Waits et al. 1999; 2–5 Ma, Yu et al. 2004; 1.6–3.0 Ma, Lindqvist et al. 2010), more recent than others (4–5 Ma, Miller et al. 2012; 5 Ma, Goldman et al. 1989; 5.5–6.7 Ma, Yu et al. 2007), and older than others (0.6–1.4 Ma; Hailer et al. 2012).

This range of divergence times for bears indicates that molecular clock estimates vary depending on the loci, calibrations, mutation rates, and models of molecular evolution considered (Pulquério and Nichols 2007; Galtier et al. 2009; Tamura et al. 2012; Warnock et al. 2012). Therefore, our estimates of polar–brown bear divergence times are not definitive values, but with other extensive nuclear DNA data (Yu et al. 2004; Hailer et al. 2012; Miller et al. 2012; Cahill et al. 2013) suggest that polar bears have been a separate lineage from brown bears over at least the past 1–2 million years of the Pleistocene and Holocene epochs. This indicates that polar bears have survived several previous warm and cool geological periods as discussed by others (Hailer et al. 2012; Miller et al. 2012), which should be incorporated into models regarding the species' response to future climate changes (e.g., Amstrup et al. 2008; Durner et al. 2009).

The genetic distances and PCoA analysis (Figure 4) of 162 SNP show black, brown, and polar bears are genetically

differentiated, and showed intraspecies geographic variation among the brown bears and black bears as with other genetic markers (Paetkau and Strobeck 1994; Talbot and Shields 1996b; Paetkau et al. 1997, 1998a, 1998b; Waits et al. 1998; Shields et al. 2000; Jackson et al. 2008; Proctor et al. 2012), and that polar bears in the southern Beaufort and Chukchi seas are not differentiated, as reported previously for microsatellites (Paetkau et al. 1999; Cronin et al. 2006) and SNP (Miller et al. 2012). Development of larger numbers of SNP will allow high resolution of population differentiation of these species.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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