

# ECOLOGICAL GENETIC STUDIES OF BEARS USING MICROSATELLITE ANALYSIS

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**Abstract:** We developed primers for typing microsatellite markers in American black bears (*Ursus americanus*). It was demonstrated that these primers can be used to type individuals from all bear species. Over 1,000 individuals from 8 species of bears (Ursidae) were typed to assess the efficacy of microsatellite analysis for studying several types of ecological genetic questions. In most populations, typing with a series of microsatellite markers provided ample resolution to identify individuals and parent-offspring relationships. Microsatellites were also highly effective for measuring genetic variation within populations and the degree to which populations are genetically distinct from each other. These results demonstrate the power of microsatellite analysis as a genetic tool for studying individual and population level relationships in bears.

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**Key words:** bears, DNA fingerprinting, microsatellites, paternity, population genetics, *Ursidae*.

The study of molecular genetics in natural populations can be divided into evolutionary and ecological time scales. Evolutionary studies address the divergence of groups of organisms over time, whereas ecological studies focus on how individuals and groups of individuals relate to and interact with one another on an ongoing basis. In bears and many other large mammals, most genetic studies have addressed evolutionary problems, including the evolutionary relationships between species and the identification of distinct evolutionary groups within species. Some ecological problems that have been under represented in genetic studies of bears include identification of individuals, study of familial relationships, and measurement of genetic diversity within populations and gene flow between populations.

The primary reason that these ecological-scale questions have not been taken up has been the lack of genetic markers sufficiently variable to be informative on this time scale. For example, studies using restriction digests of mitochondrial DNA or allozymes detected little genetic variation and failed to identify significant genetic differences between populations of North American bears (Allendorf et al. 1979, Manlove et al. 1980, Larsen et al. 1983, Cronin et al. 1991, Shields and Kocher 1991). Direct sequencing of mitochondrial DNA may yield more information on the population level, but more sensitive markers are required if individual-level questions are to be addressed.

One group of highly variable genetic markers that have great potential for studying the ecological genetics of large mammals are microsatellites (Bruford and Wayne 1993, Queller et al. 1993). Microsatellites are anonymous nuclear markers that consist of tandemly repeated short (1-6 base pair) sequence motifs. Variation at these mark-

ers consists of differences in the number of tandem copies of these sequence motifs that are present in any particular copy. The typing of individuals with highly variable genetic markers such as microsatellites is commonly referred to as DNA fingerprinting because of the ability of such markers to distinguish between individuals with a high degree of probability.

Microsatellite analysis offers certain advantages over other DNA fingerprinting methods. First, the abundance of such sequences in eukaryotic genomes (Tautz and Renz 1984) makes it relatively easy to develop a suite of microsatellite markers in previously unstudied species. Second, the detection system uses the polymerase chain reaction (PCR) lowering requirements on the quality and quantity of DNA needed for analysis. Finally, microsatellite markers are codominant (both alleles at a particular marker are visible and identifiable) and all alleles can be unambiguously resolved. Other DNA fingerprinting techniques, such as the original method described by Jeffreys et al. (1985), do not have these advantages and can be difficult to understand genetically and more complicated to analyze mathematically.

We developed microsatellite markers for bears and explored their utility for investigating the relationships of populations and individuals. This article describes progress to date and includes a review of both published data and ongoing projects. The data for polar bears (*U. maritimus*; Paetkau et al. 1995) and parts of the brown (*U. arctos*; Craighead et al. 1995) and black bear (Paetkau and Strobeck 1994) data sets have been published previously.

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## METHODS

The microsatellite markers used were developed from an American black bear genomic library as described previously (Paetkau and Strobeck 1994). Individuals were typed using either previously described radiometric (Paetkau and Strobeck 1994) or fluorescent (Paetkau et al. 1995) detection systems.

All DNA samples were collected by others, usually during independent field studies. The primers developed to amplify microsatellites were tested on each of the 8 species of bears as well as on species from several related groups including pinnepeds, procyonids, mustelids, and canids. Pedigree analysis was performed on captive bears with known pedigrees including spectacled bears (*Tremarctos ornatus*), Asiatic black bears (*U. thibetanus*), and sun bears (*U. malayanus*). Population surveys involved the typing at 8 independent microsatellites of 994 individuals from 22 populations representing the 3 North American species of bears (Fig. 1). Polar bear populations were defined according to the most recent definitions of the World Conservation Union Species Survival Commission (IUCN/SSC; IUCN/SSC Polar Bear Specialist Group 1995), but brown and black bears populations were defined by the boundaries of the study area in which they were obtained and may not have biological significance.

Genetic diversity within populations was quantified using 5 statistics (Appendix). Observed number of alleles ( $\bar{A}$ ) and expected heterozygosity ( $\bar{H}$ ) are commonly used measures of genetic variation. Probability of identity [ $P(ID)$ ] indicates the power these markers have to identify individuals. The last 2 statistics provide a measure of the ability to identify parent-offspring relationships with [ $P(PEx)$ ] and without [ $P(P-OEx)$ ] prior knowledge of the other parent's genotype. Probability of identity and the parent-offspring exclusion statistics assumes that the individuals being compared are not related, random mating within the population, and linkage equilibrium between loci. For these reasons the actual

performance of the markers may be slightly worse than suggested by the statistics.

The homogeneity of allele distributions in neighboring populations was tested using a  $G$ -test (Sokal and Rohlf 1981), and genetic differences between a selection of populations were quantified using Nei's standard genetic distance (Nei 1972). For polar bear populations, the likelihood of each individual's genotype occurring in each population was calculated as described in Paetkau et al. (1995) and plotted for certain pairs of populations.

## RESULTS AND DISCUSSION

### Range of Primer Utility

The range of species over which PCR primers are likely to work is an important consideration when deciding whether to invest time and resources in developing new microsatellite markers. Once markers are developed it is also important to inform researchers studying related species (Schlötterer et al. 1991). In general the microsatellite primers developed in American black bears give clean, variable PCR products across the family Ursidae and can be applied to all these species without modifying methodology. By comparison, we were unable to find more than a single marker that was informative in any species outside the Ursidae.

### Identification of Individuals

DNA fingerprinting methods earn their name from the fact that the markers employed are so highly variable that no 2 individuals are expected to have the same genotype. The uniqueness of individuals can be quantified using the probability of identity statistic. This statistic is derived using genotypic data from a sample of individuals in a given area and represents the expected frequency with which 2 unrelated individuals drawn randomly from the population would be expected to be identical.

The 8-locus probability of identity in the populations surveyed ranges from 1 in 93 in brown bears from Kodiak Island to <1 in 4 billion in each of 3 continental populations of black bears (Table 1). The power of resolution in any population can be increased through the use of additional markers, but logistical constraints will limit resolving power in severely depauperate populations.

This ability to identify individuals is useful in forensics where multiple samples suspected to be from the same individual need to be matched. The 8 loci used here provide ample resolving power for this purpose in most of the populations studied. This technique can also match multiple samples obtained in the field and, combined with

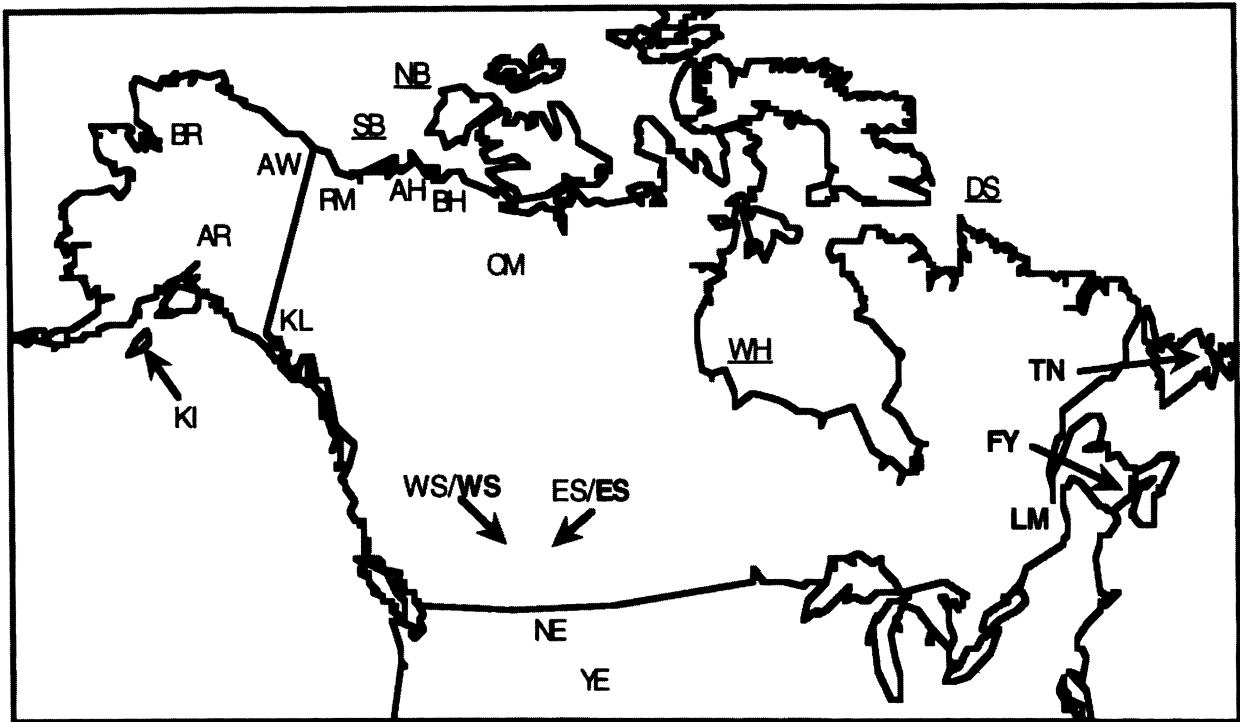


Fig. 1. Map of northern North America showing approximate sampling locations for BROWN, black, and POLAR bear populations. Brown bear populations: Alaska Range (AR), Anderson–Horton (AH), Arctic National Wildlife Refuge (AW), Brock–Hornaday (BH), Coppermine (CM), Eastern Slopes of the Canadian Rockies (ES), Kluane National Park (KL), Kodiak Island (KI), Northern Continental Divide Ecosystem (NE), Richardson Mountains (RM), Western Brooks Range (BR), Western Slopes of the Canadian Rockies (WS), Yellowstone Ecosystem (YE). Black bear populations: Eastern Slopes of the Canadian Rockies (ES), Fundy National Park (FY), La Mauricie National Park (LM), Terra Nova National Park (TN), Western Slopes of the Canadian Rockies (WS). Polar bear populations: Davis Strait–Labrador (DS), North Beaufort Sea (NB), South Beaufort Sea (SB), Western Hudson Bay (WH).

the ability to use hair (Taberlet and Bouvet 1992) and scat (Höss et al. 1992) as sources of DNA, makes it possible to survey populations without handling animals. We have obtained microsatellite genotypes using hairs collected from immobilized bears and hairs collected using scent-baited barbed wire enclosures (Woods et al. 1996).

### Family Relationships

Once genotypes are obtained for a number of individuals it is possible to identify parent–offspring relationships. In many field studies of bears, information is available on mother–offspring relationships, and it would be useful to identify fathers. In the populations surveyed, the 8-locus probability of parental exclusion—the probability that, given a cub with 1 parent who’s genotype is known, a random individual who is not the other parent will be excluded as the other parent—ranges from 0.6603 in Kodiak brown bears to 0.9997 in 2 populations of black bears.

This approach can be extended to situations where parent–offspring relationships are not known, although resolving power will be reduced. The 8-locus probability of parent–offspring exclusion—the probability that any 2 random, unrelated individuals can be genetically excluded as parent and offspring—ranges from 0.4385 to 0.9936 in the populations studied. Combining these approaches should permit the reconstruction of pedigrees in well sampled, genetically diverse populations, and could make it possible to study questions such as dispersal from natal home ranges for animals on which no field data on relationships are available.

The first application of this approach in bears was a study of male productivity in brown bears from the Western Brooks Range in Alaska (Craighead et al. 1995, Craighead et al. 1998). This population had been studied continuously for 16 years, and a large proportion of the animals in the study area had been sampled. Of 57 offspring from 30 known family (mother–offspring) groups, fathers were identified for 36 individuals, and

**Table 1. Measures of genetic diversity in various populations of North American bears as measured using 8 highly variable microsatellite markers. The measures are: mean observed number of alleles ( $\bar{A}$ ), mean expected heterozygosity ( $\bar{H}$ ), probability of identity [ $P(ID)$ ], probability of parental exclusion [ $P(PEx)$ ], and probability of parent-offspring exclusion [ $P(P-OEx)$ ].**

Species	Population (2N)	$\bar{A}$	$\bar{H}$	$P(ID)$ (inverse)	$P(PEx)$	$P(P-OEx)$
Brown	BR (306)	7.63	0.749	140,000,000	0.9981	0.9736
Brown	AW (30)	5.75	0.737	48,000,000	0.9972	0.9669
Brown	RM (238)	7.50	0.756	290,000,000	0.9987	0.9808
Brown	AH (44)	5.38	0.667	2,100,000	0.9883	0.9163
Brown	BH (112)	5.75	0.650	3,800,000	0.9919	0.9348
Brown	CM (76)	5.75	0.600	780,000	0.9856	0.9042
Brown	KI (68)	2.13	0.265	93	0.6603	0.4385
Brown	AR (174)	7.75	0.733	49,000,000	0.9970	0.9640
Brown	KL (102)	7.38	0.762	260,000,000	0.9986	0.9791
Brown	ES (66)	6.38	0.656	6,300,000	0.9940	0.9421
Brown	WS (44)	5.63	0.685	4,800,000	0.9917	0.9276
Brown	NE (102)	6.75	0.703	13,000,000	0.9945	0.9454
Brown	YE (108)	4.38	0.555	152,000	0.9713	0.8461
Black	WS (106)	8.50	0.809	4,300,000,000	0.9996	0.9917
Black	ES (64)	8.75	0.819	7,200,000,000	0.9997	0.9932
Black	LM (64)	8.63	0.819	7,500,000,000	0.9997	0.9936
Black	FY (22)	5.13	0.728	16,000,000	0.9951	0.9495
Black	TN (46)	2.63	0.428	1,300	0.8100	0.5699
Polar	SB (44)	5.75	0.641	4,700,000	0.9936	0.9399
Polar	NB (60)	6.38	0.643	3,000,000	0.9913	0.9232
Polar	DS (52)	5.88	0.610	990,000	0.9867	0.8983
Polar	WH (60)	5.38	0.626	1,300,000	0.9873	0.8982

multiple paternity within litters was shown to be common.

Pedigree information is also important for keeping breeding records of captive-bred individuals. We have been using up to 14 microsatellite loci to confirm the pedigrees of captive-bred animals from non-North American bear species. A total of 50 individuals from 11 families of spectacled, sun, and Asiatic black bears have been typed to date. Population samples were not available for these species, but the amount of variation present in these animals is illustrated by 1 family group of 9 sun bears in which a mean of 4.6 alleles were observed at the 13 loci used to type these individuals. This level of variation appears to be sufficient in practice to resolve ambiguities in pedigrees. Similar results have been reported in the giant panda (*Ailuropoda melanoleuca*) where uncertainties regarding paternity in 1 pedigree were resolved using a suite of microsatellite loci developed in that species (Zhang et al. 1994).

Two sources of confusion in the use of microsatellites for pedigree analysis are mutations and segregating 'null' alleles, both of which have been seen at loci used here (Craighead et al. 1995, Paetkau and Strobeck 1995). In addition, the study by Craighead et al. (1995) provides

an empirical demonstration that the number of loci needed to resolve relationships can be restrictive; one situation was found where 2 males (possibly full siblings) could not be genetically excluded as fathers of a particular cub despite knowledge of the mother's genotype and a theoretical probability of parental exclusion of 0.9981 in this population. Studies in less genetically diverse populations and those where maternal genotypes are not known will require careful consideration of the number of loci employed. The logistics of studying relationships more distant than parent-offspring are likely to be prohibitive at this time.

### Genetic Diversity within Populations

A major thrust in conservation genetics is the threat that loss of genetic diversity may pose to the survival of small isolated populations (Franklin 1980, Soulé 1980, Lacy 1993). Long-term concern focuses on the preservation of biological diversity; specifically, maintaining the ability of populations to evolve in the face of a changing environment. Short-term concerns center on the fact that rapid and dramatic loss of diversity can result in inbreeding depression, exacerbating the anthropogenic threats that so many populations are currently facing.

The amount of neutral genetic variation maintained in a population is a function of the opposing forces of genetic drift and the introduction of new variation through mutation and immigration (Hartl and Clark 1989). Genetic drift is the process by which genetic variation is lost in finite populations through the sampling of each generation's genes from the previous generation's gene pool. Since this sampling is random, some variants of particular genes will inevitably be missed in every generation resulting in their permanent loss from the gene pool. The larger the population size, the more slowly genetic drift occurs and the more genetic diversity is maintained in the population.

Clearly fears about loss of diversity cannot be well understood without accurate and sensitive measures of diversity. Nuclear genetic markers are indispensable for such measurements as the vast majority of genetic material is in the nucleus, and it is the loss of nuclear diversity that leads to inbreeding depression. Furthermore, life history traits such as limited female dispersal can result in maternally inherited markers showing different patterns than found with nuclear markers. Another consideration is that any genetic study of populations should be based on a number of independent markers because the forces that affect the amount of diversity maintained in a population are stochastic in nature, making it necessary to combine data from several markers to arrive at a meaningful estimate. Finally, monitoring genetic diversity in species with inherently low diversity, or populations that have already experienced significant losses, requires the sensitivity of highly variable markers. These considerations suggest that microsatellites should be useful markers for measuring genetic diversity.

Measures of microsatellite diversity in the 22 populations of North American bears surveyed here give an excellent picture of how populations and species differ in their levels of genetic diversity (Table 1). Results from non-insular populations indicate that polar bears are less genetically diverse than brown bears, which in turn are less diverse than black bears. This pattern may relate to ecological differences between these species, but is more easily explained by differences in their abundance; there are more than 500,000 American black bears, whereas the population estimate for brown bears in North America is in the range of 50,000 (Servheen 1990), and the global estimate for polar bears is around 25,000 (IUCN/SSC Polar Bear Specialist Group 1995).

In insular populations, including Kodiak brown bears and Newfoundland black bears, dramatically reduced levels of diversity are seen relative to continental populations. By comparison, populations that have become

isolated due to habitat loss, such as Yellowstone brown bears and black bears from Fundy National Park, have lost some genetic diversity, but the losses have not yet been dramatic (Waits et al. 1998). Comparisons of anthropogenic impact can also be made between species. For example, Eastern Slope brown bears show a decline in diversity relative to northern populations, whereas black bears from the same area are among the most genetically diverse populations seen to date. An interesting decline in diversity is also seen in brown bears on the Barren Grounds of the Northwest Territories (NWT) as one proceeds from west to east toward the edge of the species range. Not only are such baseline data important in order to monitor future changes, knowledge of diversity on natural islands should allow predictions to be made about the genetic future of populations in anthropogenic habitat islands.

## Population Structure

In addition to measuring the amount of genetic diversity within populations, it is important to know how genetically distinct populations are. Because bears are capable of moving long distances, geographically close populations might be expected to be genetically homogenous. On the other hand, long-term field studies typically reveal that most bears are at least seasonally philopatric. Direct genetic studies offer the only practical way to quantify gene flow between populations.

The simplest question to ask concerning the relationship between populations is whether allele distributions in 2 different populations are homogenous. In polar bears, all pairs of populations, including the adjacent populations in the Beaufort Sea, have significantly different allele distributions. In all pairs of brown and black bear populations for which calculations were made, allele distributions are significantly non-homogenous. This includes the Anderson-Horton and Brock-Hornaday populations of Barren Ground grizzlies with geographic centers <150 km apart and recorded exchange of both males and females (P. Clarkson, Gwich'in Renewable Resour. Board, Inuvik, NWT, pers. commun., 1995). These results clearly show that gene flow in the 3 North American species of bears is not sufficient to cause genetic uniformity, even between areas that are within the range over which animals have been observed to disperse.

We used Nei's genetic distance to quantify genetic differences between populations. In polar bears, concordance is seen between Nei's genetic distance and geographic separation. In 5 populations of Arctic grizzly bears, arranged approximately east to west across northern Alaska, Yukon, and NWT, there is excellent agree-

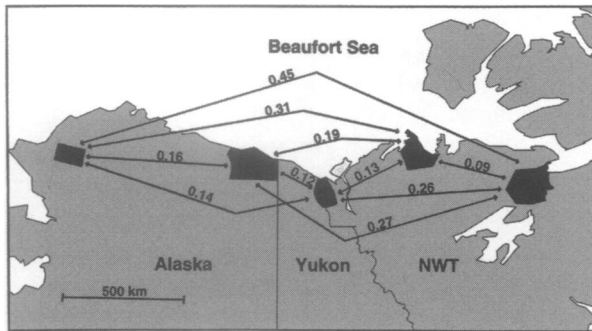


Fig. 2. Nei's genetic distance between 5 Arctic grizzly bear populations. Although the values do not correlate strictly with geographic distance, they illustrate the ability of microsatellite data sets to detect population genetic structure.

ment between geographic separation and genetic distance (Fig. 2). In more widely separated populations, however, genetic distances get so large that they are in the range of between-species values. For example, the genetic distance between Western Brooks Range brown bears and brown bears from the Eastern Slopes of the Canadian Rockies is 0.50, not dramatically less than the value of 0.64 between Western Brooks Range brown bears and *black bears* from the Eastern Slopes. Other distance measures that specifically account for the mutational mechanisms of microsatellites may be able to enhance or extend the population structure signal in microsatellite data sets, but ultimately the high mutation rate and constraints on allele size will set an upper limit to the comparisons that can be made (Goldstein et al. 1995).

If populations are sufficiently distinct genetically, it should be possible to determine where an individual came from based on its genotype. The degree to which one is able to make such determinations can also be used on its own to describe how distinct populations are. A test has been developed in which the expected frequency of each individual's genotype is calculated based on observed allele frequencies in 2 or more populations. Individuals are then assigned to the population where the likelihood of their genotype's occurrence is highest, and this serves as a measure of population differentiation (Fig. 3a, Paetkau et al. 1995). In polar bears it was found that 94% of individuals were assigned to the correct side of the Canadian Arctic, although assignments between neighboring populations were only correct in 60% of cases. Ultimately, the use of a large number of markers may make it possible to identify immigrant individuals.

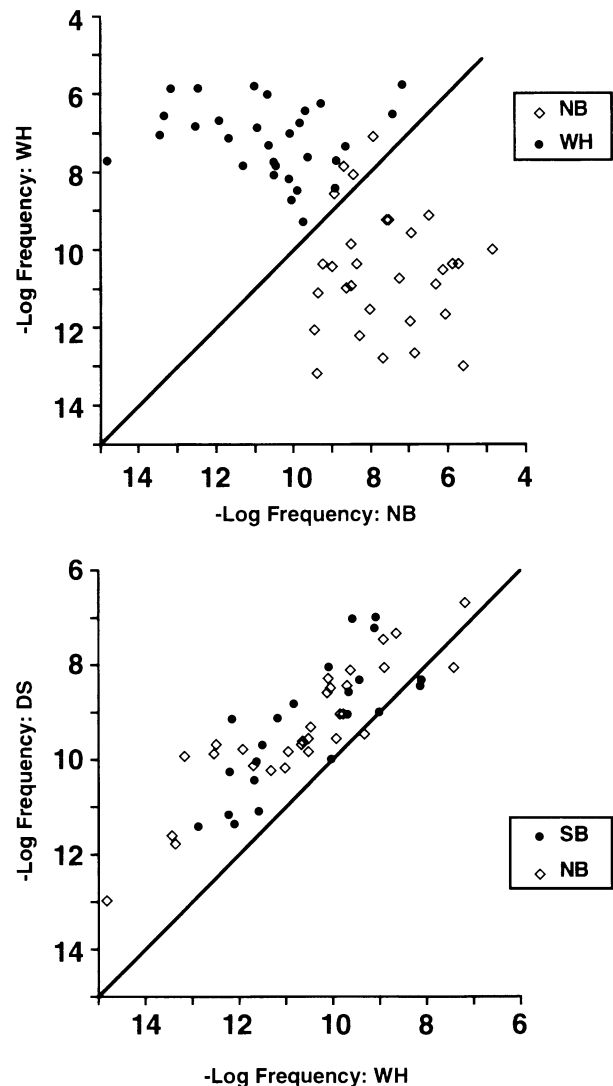


Fig. 3. The expected frequency of the genotype of each individual typed in a population was calculated based on observed allele distributions in 2 populations. (A) For individuals from the North Beaufort Sea (NB) and Western Hudson Bay (WH) populations, the expected frequency of an individual's genotype is generally much higher in the population in which that individual was sampled demonstrating that the region of origin for a given sample can be predicted. (B) The genotypes of individuals sampled in the South (SB) and North Beaufort Sea are generally more likely to occur in Davis Strait-Labrador (DS) than in WH. This approach can reveal genetic relationships and patterns of gene flow between populations.

Once genetic relationships between populations have been quantified, this information can be used to study patterns of gene flow between regions. In polar bears, all genetic measures indicated that gene flow between

populations in the western Arctic and Hudson Bay is through populations on the east side of Baffin Island (Fig. 3b). This approach has not yet been extended to other species.

All the approaches to population structure mentioned above start with pre-defined populations and ask how they differ from one another. An alternate approach is to take samples from across a range and ask if there are any natural discontinuities that might be used as the basis for a biologically founded population definition. Unfortunately, statistical methods are not yet available for this approach.

## CONCLUSIONS

Microsatellites are highly variable, codominant, Mendelian, nuclear genetic markers that are becoming widely used in the areas of molecular ecology and population genetics. Microsatellite markers developed for American black bears can be used in all bear species. The typing of a large number of individual bears from the 3 North American species of bears demonstrated that these markers can be used successfully in studies of identity, relationship, and population structure. The availability of these markers opens the doors to studies of the ecological genetics of bears that have not previously been feasible, and should enhance both our basic understanding of bear biology and our ability to manage bear populations effectively.

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## APPENDIX

Formulas for average expected heterozygosity ( $\tilde{H}$ ; corrected for small sample size; Nei and Roychoudhury 1974), probability of identity [ $P(ID)$ ], probability of parental exclusion [ $P(PEX)$ ] (Chakravarti and Li 1983), and probability of parent–offspring exclusion [ $P(P-OEX)$ ], where  $n$  is sample size (number of chromosomes),  $a_k = \sum_i p_i^k$ , and  $p_i$  is the observed frequency of the  $i$ th allele.

$$\tilde{H} = 1 - (na_2 - 1)/(n - 1)$$

$$P(ID) = 2(a_2)^2 - a_4$$

$$P(PEX) = 1 - 2a_2 + a_3 + 2a_4 - 3a_5 + 3a_2a_3 - 2(a_2)^2$$

$$P(P-OEX) = 1 - 4a_2 + 4a_3 - 3a_4 + 2(a_2)^2$$