Behav Ecol Sociobiol (1992) 30:83–94

Behavioral Ecology and Sociobiology

# A study of the genetic relationships within and among wolf packs using DNA fingerprinting and mitochondrial DNA

Niles Lehman[1,*], Peter Clarkson[2], L. David Mech[3], Thomas J. Meier[4], and Robert K. Wayne[1]

[1] Department of Biology, University of California, Los Angeles, CA 90024, USA
[2] Department of Renewable Resources, Govt. of the Northwest Territories, Inuvik, N.W.T. Canada X0E OTO
[3] U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, MD 20702, USA (mailing address: North Central Forest Experiment Station, 1992 Folwell Ave., St. Paul MN 55108, USA)
[4] U.S. National Park Service, P.O. Box 9, Denali National Park, AK 99755, USA

Received May 10, 1991 / Accepted October 17, 1991

**Summary.** DNA fingerprinting and mitochondrial DNA analyses have not been used in combination to study relatedness in natural populations. We present an approach that involves defining the mean fingerprint similarities among individuals thought to be unrelated because they have different mtDNA genotypes. Two classes of related individuals are identified by their distance in standard errors above this mean value. The number of standard errors is determined by analysis of the association between fingerprint similarity and relatedness in a population with a known genealogy. We apply this approach to gray wolf packs from Minnesota, Alaska, and the Northwest Territories. Our results show that: (1) wolf packs consist primarily of individuals that are closely related genetically, but some packs contain unrelated, non-reproducing individuals; (2) dispersal among packs within the same area is common; and (3) short-range dispersal appears more common for female than male wolves. The first two of these genetically-based observations are consistent with behavioral data on pack structure and dispersal in wolves, while the apparent sex bias in dispersal was not expected.

## Introduction

The use of hypervariable minisatellite probes, which detect variability at variable number of tandem repeat (VNTR) loci and produce "genetic fingerprints", has allowed the precise testing of paternity in animal populations and has led to a flourish of recent studies that have questioned some conclusions based on behavioral observations (reviewed in Bruke 1989). However, uncertainty exists about the utility of genetic fingerprinting analyses for deducing patterns of relatedness among individuals living in the same area or in a single social group (Lynch 1988). Even an assessment of paternity requires a detailed knowledge of the study population and DNA samples from parents and their possible offspring (c.f. Wetton et al. 1987).

Nevertheless, data correlating genetic relatedness of individuals with behavior in populations are essential to test hypotheses about inclusive fitness (Hamilton 1964), and genetic fingerprinting provides researchers with the ability to assess the amount of sharing of a large number of highly variable alleles within a study population. For example, Packer et al. (1991) described patterns of similarity based on genetic fingerprint profiles in lion prides and showed that genetic fingerprinting could be used to define groups of individuals that were related at the level of siblings or parent-offspring. Yet Packer et al. (1991) needed accurate pedigrees of the studied populations as well as the history of each sampled lion pride to assess the degree of bandsharing relatedness among siblings and parent-offspring. Rarely are these parameters known in populations under study, so it would be desirable to infer relationships based primarily on molecular-genetic data.

In this report, we use the combination of fingerprint and mitochondrial DNA (mtDNA) data to classify individuals as unrelated, moderately related, or closely related at the level of siblings or parent-offspring. Our method does not require detailed knowledge about the study populations but relies instead on an extrinsic calibration of relatedness. We apply this approach to three pack clusters of the gray wolf (*Canis lupus*) and test the following hypotheses based on observations of social behavior: (1) wolf packs are usually composed on an unrelated breeding male and female and their offspring; (2) dispersal among nearby packs is common and; (3) dispersal is not sex-biased.

### *The approach*

We first document the relationship between fingerprint similarity (bandsharing) and the coefficient of related-

* *Present address:* Dept. of Molecular Biology, Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

*Offprint requests to:* N. Lehman at the present address

ness ($r$) in two groups of captive individuals. We compute the distance ($D$) in standard errors above the mean similarity of unrelated individuals to the lower bound of the 95% confidence interval spanning the similarity values among siblings or parent-offspring. A second, more stringent $D$ value is calculated to the lower similarity bound that includes only individuals related as siblings or parent-offspring. These two values provide a means of classifying individuals of unknown relationships if the average similarity among unrelated individuals is known for each population.

To determine the average fingerprint similarity among unrelated individuals in wild populations, we assess the similarity among a group of individuals that, based on independent genetic data, are likely to be unrelated. To do this, we assay the population for mitochondrial DNA (mtDNA) variation and define distinct genotypes. We then compute the average similarity among individuals from different packs with distinct mtDNA genotypes. As a consequence of the strict maternal and clonal inheritance of mammalian mtDNA, such pairwise comparisons must be among individuals with different mothers and these individuals cannot be related more closely than half-sibs ($r=0.25$) unless they are a father-offspring pair. In the gray wolf, reproduction is predominantly performed by a single breeding male and female pair (Mech 1970). Thus, individuals who are not of the same pack and who have differing mtDNA genotypes are unlikely to be closely related, and the average fingerprint similarity among such individuals can serve as a baseline from which $D$ values can be computed. We then classify pairs of wild-caught individuals as being unrelated ($r<0.1$), moderately related (related at or near the level of first cousins, $0.5>r>0.1875$), or closely related (related at the level of siblings or parent-offspring, $r\approx 0.5$) based on the $D$ values that the comparisons generate.

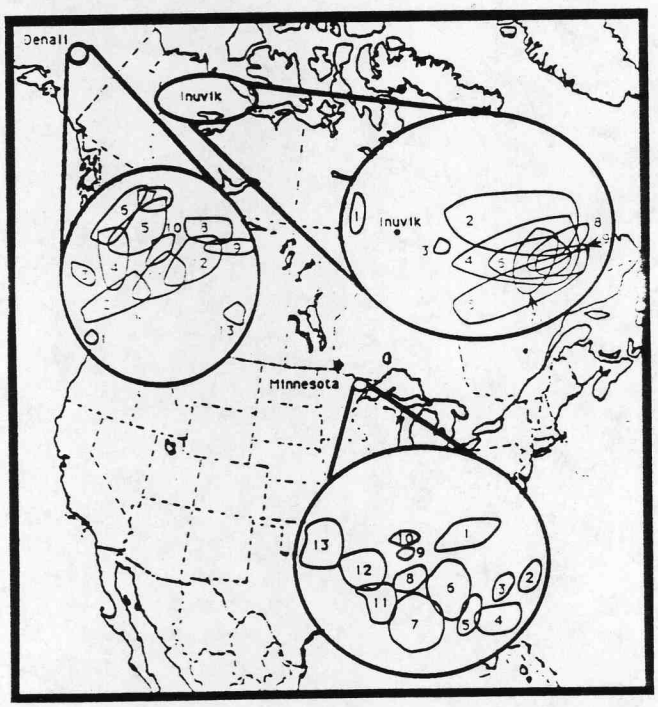



Fig. 1. A map showing the locations of pack clusters and the spatial relationships of packs in 1989–1990. Pack identification is by number as follows. *Minnesota* pack cluster: *1*, Emerald; *2*, Kawishiwi; *3*, Isabella Lake; *4*, Sawbill; *5*, Pike; *6*, Little Gabbro; *7*, Nip Creek; *8*, Birch; *9*, Ely; *10*, Winton; *11*, Perch Lake; *12*, Bear Island; *13*, Tower. *Denali* pack cluster: *1*, Chedotlothna; *2*, McLeod Lake; *3*, Highpower; *4*, Foraker; *5*, McKinley River; *6*, Birch Creek; *7*, Chitsia; *8*, Stampede; *9*, Ewe Creek; *10*, Little Bear; *11*, Clearwater; *12*, East Fork; *13*, Windy Creek. *Inuvik* pack cluster: *1*, Rat River; *2*, Island; *3*, Williams Lake; *4*, Wolverine; *5*, Iroquois; *6*, Ridge; *7*, Charlotte's; *8*, Rendezvous; *9*, Anderson River

### *The experimental system*

Gray wolves generally form monogamous pairs whose offspring may remain in the pack for a few years and contribute to the rearing of future offspring (Mech 1970; Kleiman and Eisenberg 1973; Macdonald and Moehlman 1982; but for exceptions see Harrington et al. 1982). Wolf packs of 2–30 individuals develop and defend territories from invasion by other individuals or packs (Mech 1973; Peterson 1977). Individuals that choose to disperse may travel long distances, hundreds of kilometers in some cases, with many wolves in southern areas dispersing 50 km or more (Mech 1987; Gese and Mech 1991). However, dispersing wolves often colonize areas near their natal pack territories (Fritts and Mech 1981; Gese and Mech 1991). It seems unlikely that offspring would disperse to other packs because inter-pack aggression can be severe (Mech 1970; Van Ballenberghe 1983). Dis-

Table 1. Behavioral hypotheses tested by genetic data

| Hypothesis | Evidence for rejection |
|---|---|
| | *mtDNA* |
| 1. Packs composed of an unrelated breeding pair and their offspring | a. Third genotype in pack<br>b. Two or more females with different genotypes<br>c. Two or more males with genotypes not found in females |
| | *Genetic Fingerprinting* |
| | d. High similarity between putative breeding pair<br>e. Low similarity between same sexed wolves or between more than one pairs of wolves |
| | *Genetic Fingerprinting* |
| 2. Dispersal uncommon between neighboring or overlapping packs | a. High similarity ($D>2.29$) among wolves from neighboring or overlapping packs |
| | *Genetic Fingerprinting* |
| 3. Long-range dispersal not sex biased | a. Non-random distribution of male and female connections between packs ($D>1.52$)<br>b. Male-male and female-female inter-pack average similarities unequal |

persal tendency is not believed to be biased according to sex (Mech 1987; Gese and Mech 1991), and studies on Minnesota wolves have failed to detect a consistent sex bias in average dispersal distance (Mech 1987; Fuller 1989; Gese and Mech 1991).

These social characteristics have implications for the expected pattern of genetic relatedness in populations. Within most packs, individuals should be related as parent-offspring or siblings except for the reproducing pair. Thus, for within-pack comparisons, only one intra-pack VNTR bandsharing similarity value should be less than a threshold number of standard errors above the mean similarity of unrelated individuals, and this low value should not be between members of the same sex (Table 1). Secondly, within packs usually a maximum of two mtDNA genotypes should be found, females should not have different genotypes, and two males should not share the same genotype if it is different from that found in female members of the pack (Table 1). Between-pack comparisons should in general not show high values of similarity if offspring most commonly disperse out of the neonatal area. Moreover, inter-pack similarities should not show a significant sex bias if both sexes are equally likely to disperse a long distance and reproduce equally. In this study we test these predictions using mtDNA and genetic fingerprinting analyses of gray wolves from 13 packs in northern Minnesota, 13 packs in Denali National Park, Alaska, and 9 packs from an area near Inuvik, Northwest Territories.

## Methods

*Samples.* The individuals used in this study are from three wild wolf pack clusters (Fig. 1) and two captive wolf colonies. Summary information on the wild pack clusters is provided in Table 2. It is important to emphasize that we most often analyzed only a few individuals within each wild pack. An additional goal of our study is to demonstrate that useful information about population structure can be gleaned from even a limited sampling of individuals.

*The Minnesota packs.* The 42 wolves from this area belong to 13 distinct packs clustered in an area encompassing about 2200 $km^2$ within the Superior National Forest in the northeast portion of Minnesota. The territory of each pack is well defined, with little or no overlap (Fig. 1). These packs have been studied by L.D.M. for 22 years. Pack sizes fluctuate from year to year but averaged 5–6 wolves during the study period. One to six individuals per pack were included in our analysis. From the Birch, Ely, Emerald, Isabella Lake, and Sawbill packs, one of the putative breeding adults was sampled, and from the Pike Lake pack both putative breeding adults were sampled.

*The Denali packs.* The 22 individuals in 13 wolf packs are from Denali National Park and Preserve in central Alaska and have been under study by L.D.M. and T.J.M. since 1986. Pack sizes averaged 8–9 wolves during the study period. Between one and three individuals from these packs, which range across approximately 17,000 $km^2$, were included in our analysis. Portions of many of the pack territories overlap, and the separation of packs is less distinct than in the Minnesota pack cluster (Fig. 1). From the Chitsia, East Fork, Ewe Creek, Foraker, McKinley, Stampede, and Windy Creek packs, one of the putative breeding adults was sampled, and from the Clearwater pack both putative breeding adults were sampled.

*The Invuik packs.* The 46 wolves from this area come from nine packs studied by P.C. since 1987 and range across approximately 150,000 $km^2$. These wolf packs are from a large expanse of tundra/forest habitat in the vicinity of the community of Inuvik, in northwestern Northwest Territories, Canada. Except for the Rat River and Williams Lake packs, the ranges of all the packs overlapped extensively during 1987–1990 (Fig. 1). Pack sizes averaged 6–8 wolves during the study period. Between two and eight individuals per pack were included in our analysis; for the Charlotte's, Iroquois, Rat River, Rendezvous, Williams Lake, and Wolverine packs all wolves belonging to the pack at the time of sampling were included. From each pack at least one of the putative breeding adults was sampled and from five packs, Island, Rat River, Rendezvous, Williams Lake, and Wolverine, both the suspected breeding male and female were sampled.

*The Julian pack.* This captive family of wolves is located in Julian, California under the care of P. Kinnis. We obtained samples from each member of a breeding pair and 15 of their offspring in order to assess the allelic similarities between individuals with a known coefficient of relatedness of $r = 0.5$.

*The Forest Lake colony.* This captive group of wolves is located near Forest Lake, Minnesota under the care of T.J. Kreeger. The colony was founded in 1960 from individuals believed to be unrelated. The colony includes individuals from a large pedigree of wolves (Packard et al. 1983) with relationships ranging from siblings of sib-sib matings to second cousins and unrelated individuals. Because all of the relationships between individuals are known with a high degree of certainty as a result of careful observation (wolves were paired in pens for mating), and because many involve consanguineous matings, the 19 individuals chosen for analysis provide an opportunity to assess allelic similarities between individuals with varying levels of $r$, ranging from essentially 0 to 0.75. Six distinct classes of $r$-value comparisons were possible (Fig. 2).

*DNA preparation.* Genetic analysis was performed on DNA extracted from blood samples taken from live individuals. Individual wolves were captured between 1988 and 1990, and approximately 5–10 ml of heparinized blood were recovered from anesthetized individuals by venipuncture. White blood cells were separated from other blood components in the laboratory and then frozen until needed. DNA was extracted from white cells by standard methods (Maniatis et al. 1982).

**Table 2.** General pack information for wild wolves

| Pack cluster | Total # of packs sampled | Total # of individuals sampled | Total geographic range of packs ($km^2$) | # of wolves (# of packs) on which VNTR data were collected | Territorial overlap |
|---|---|---|---|---|---|
| Minnesota | 13 | 42 | 2,200 | 28 (13) | none |
| Denali | 13 | 22 | 17,000 | 19 (10) | moderate |
| Inuvik | 9 | 46 | 150,000 | 37 (9) | extensive |

*Mitochondrial DNA analysis.* To identify restriction-fragment length polymorphisms, genomic DNA was digested with a restriction enzyme, and the resulting fragments were separated by agarose gel electrophoresis and transferred to nylon membranes. After hybridization with a radioactively-labelled probe of the entire mtDNA genome cloned from a domestic dog, the fragments were visualized by autoradiography (Lehman et al. 1991). The fragment patterns produced by a battery of 21 restriction enzymes allowed identification of composite genotypes for each individual that could be compared within and among pack clusters and to known gray wolf genotypes world-wide (Lehman et al. 1991).

*Hypervariable DNA analysis.* DNA samples were digested with an excess of *Hin*fI restriction enzyme and electrophoresed into 1% agarose gels. The DNA was then transferred onto nylon membranes by capillary action in 10X SSC for 48 h to affect transfer of fragments larger than approximately 1 kb pairs. The membranes were then probed with the "minisatellite" probe 33.6 originally described by Jeffreys et al. (1985). For details of the hybridization process see Gilbert et al. (1990).

For each gel we used a 20-slot 1-mm comb that allowed for 19 different samples, as the first and last lanes were always duplicated. The gel loading orders for each of the three wild pack clusters were chosen to represent the packs in the most efficient manner, either by including as many packs as possible on a single gel or by including as many individuals from the same pack as were available on a single gel. This facilitated the calculation of similarity among individuals, who, based on the mtDNA analysis, were likely to be unrelated and allowed us to examine similarities within and among packs without cross comparisons between different gels.

Because different restriction enzymes may reveal different fragments within the scorable range, we could potentially sample a greater number of VNTR loci by using two restriction enzymes. Thus, most samples were assayed with both *Hin*fI and *Hae*III restriction enzymes in order to increase the number of loci surveyed and improve the power of the statistical tests applied to the VNTR data. Because only 19 individuals could be run per gel, for the Minnesota and Inuvik pack clusters more than one gel was run per enzyme. A total of nine scorable gels was produced for the wild populations (Table 3).

The resulting DNA "fingerprint" patterns were scored visually by placing the autoradiograms produced after hybridization on a lightbox and using a transparent ruler to determine the presence or absence within each lane of fragments of a particular migration distance. The ruler was kept exactly perpendicular to the migration direction by alignment with co-migrating fragments in lanes #1 and #20. Fragments within the size range of 1–12 kb were scored. The result was a presence-absence matrix of fragment possession for single pack clusters digested with a single enzyme. No attempt was made to compare fragments between gels. However, when two restriction enzymes were used to assay the same sets of individuals, a presence-absence matrix could be generated by combining the fragments scored from the two gels (Table 3).

The empirical determination of relatedness could be made by

**Table 3.** Statistics of VNTR gels on wild-caught wolves

| Gels | Enzyme used | Pack cluster | Number of individuals scored | Packs included | Within-pack statistics ($S_x \pm$SE) | Between-pack statistics ($S_x \pm$SE) | Unrelated between-pack statistics ($S_{unrelated} \pm$SE) |
|---|---|---|---|---|---|---|---|
| #1 | *Hin*fI | Minnesota | 19 | Perch Lake, Tower, Nip, Little Gabbro, Sawbill | 609.7 ± 103.3 | 381.3 ± 107.4 | 367.6 ± 108.51 |
| #2 | *Hin*fI | Minnesota | 19 | Emerald, Pike, Sawbill, Kawishiwi, Birch, Ely, Bear Island, Isabella Lake | 474.0 ± 81.1 | 390.0 ± 80.5 | 363.4 ± 129.4 |
| #3 | *Hae*III | Minnesota | 19 | Emerald, Pike, Ely, Kawishiwi, Birch, Bear Island, Isabella Lake Perch Lake, Winton, Little Gabbro | 461.2 ± 80.7 | 401.2 ± 80.5 | 426.4 ± 117.5 |
| #2 plus #3 | *Hin*fI plus *Hae*III | Minnesota | 15 | Emerald, Pike, Ely, Kawishiwi, Birch, Bear Island, Isabella Lake Perch Lake, Winton, Little Gabbro, Sawbill | 468.8 ± 80.7 | 392.8 ± 80.5 | 378.6 ± 80.7 |
| #4 plus #5 | *Hin*fI plus *Hae*III | Denali | 19 | Stampede, East Fork, Foraker, Little Bear, Birch Creek, McKinley, Chitsia, Clearwater, McLeod Lake, Highpower | 627.4 ± 80.4 | 492.1 ± 85.4 | 487.9 ± 85.3 |
| #6 | *Hin*fI | Inuvik | 18 | Rat River, Rendezvous Ridge, Charlotte's, Iroquois Anderson River, Wolverine | 708.3 ± 105.3 | 534.2 ± 119.9 | 554.2 ± 118.2 |
| #7 | *Hin*fI | Inuvik | 19 | Rat River, Island, Anderson River, Williams Lake | 739.8 ± 91.58 | 443.6 ± 110.4 | 418.3 ± 109.7 |
| #8 plus #9 | *Hin*fI plus *Hae*III | Inuvik | 17 | Ridge, Anderson River, Rendezvous, Wolverine, Island | 631.9 ± 76.5 | 494.0 ± 81.5 | 487.6 ± 80.8 |

calculating bandsharing similarity values among individuals. The similarity value, $S$, between any two individuals was calculated as:

$$S = 2n_{xy}/(n_x + n_y),$$

where $n_{xy}$ is the number of fragments shared between individuals $x$ and $y$, and $n_x$ and $n_y$ are the total numbers of fragments possessed by individuals $x$ and $y$, respectively. Other statistics used were $S_x$, the average similarity among a group of individuals, and $\bar{n}$, the average number of fragments scored per individual per gel.

The statistic $S_x$ is a biased estimator of similarities because it possesses components that are not independent. Therefore, we used a statistic for the variance in $S_x$ given by Lynch (1990) that results in an unbiased estimate of the true variance in similarity among individuals:

$$\mathrm{Var}(S_x) = 2S_x(1-S_x)(2-S_x)/\bar{n}(4-S_x).$$

Then the standard error for the $S_x$ values is calculated as $\sqrt{[\mathrm{Var}(S_x)]}$. In our analysis, all $S_x$ and SE values have been multiplied by 1000 for ease of presentation.

The mtDNA-genotype analysis provides a method of determining genetic relationships that is completely independent of the VNTR data because nuclear loci are not linked genetically to cytoplasmic loci. For each fingerprinting gel, the average value of all pairwise similarities between individuals who do not have the same mtDNA genotype ($S_{unrelated}$) could be computed as an empirically-determined baseline of similarity among unrelated individuals. Within-pack comparisons were excluded from these calculations as they would tend to bias the estimates of similarity among unrelated individuals upward if most packs are composed of highly-related individuals including father-offspring pairs that may be of differing mtDNA genotpyes. The statistic $D$ could then be defined as the number of standard errors above $S_{unrelated}$ for a given gel that the $S$ value between a particular pair of wolves falls.

To assess the significance of the difference between the average similarities within packs and the average similarities among packs, we used a permutation test appropriate for similarity data (Dietz 1983; Wayne et al. 1991). Each fingerprinting gel generated a triangular matrix of pairwise similarity values. Typically, the matrices contained 171 entries because 19 individuals can be compared on a single gel. For each matrix, we randomly subsampled the data by computer 10,000 times, each time partitioning the pairwise similarity values into two groups. One group contained the number of within-pack comparisons that the gel provided (e.g. 10), and the other group contained the number of among-pack comparisons that the gel provided (e.g. $171-10=161$). We then computed the average similarity difference between the two groups of comparisons, and contrasted this value with the observed difference between within- and among-pack similarity values on each gel.

## Results

### *Calibration of similarity values*

To determine the level of relatedness among unknown individuals, we first analyzed wolves of known genetic relationships from the two captive wolf populations. In the Julian population, all comparisons are between parents and offspring or between siblings, except for the comparison between the two breeding adults, which are presumably unrelated. Indeed, while all of the 104 $r=0.5$ comparisons range between 700 and 889 ($S_x=785.3$, $SE=56$), the two parents are measured at $S=642$. For the Forest Lake Colony, $r$ values between about 0 and 0.75 generate $S$ values ranging between 405 and 894 (Fig. 2). This range of $S$ values is similar to that of the ranges found among the wild-caught wolves (Fig. 3),

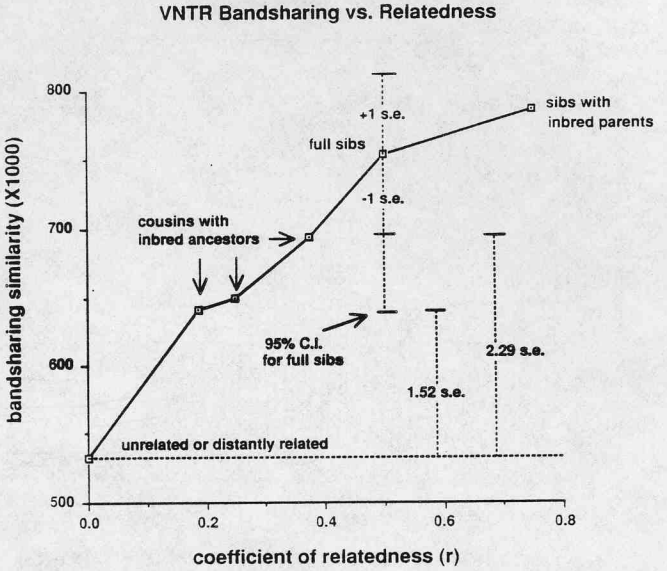
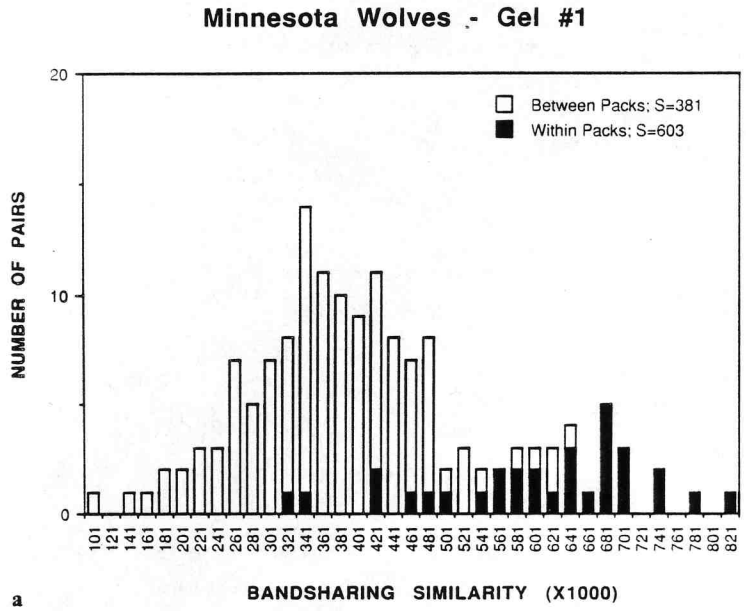
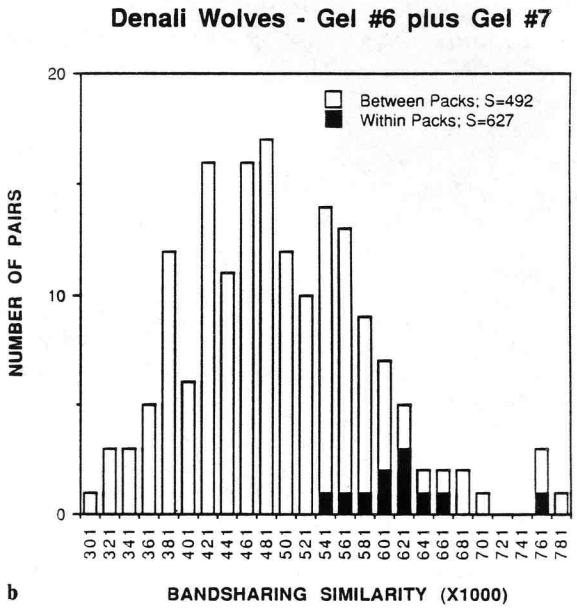



**Fig. 2.** Empirical relationship between observed levels of variable number of tandem repeat (VNTR) bandsharing and genetic relatedness in the Forest Lake captive wolf colony. Wolves that are presumably unrelated possess a level of VNTR bandsharing that is 2.29 standard errors below the range of similarity values that is generated by comparisons between full sibs or parents and offspring. Unrelated wolves also fall at least 1.52 standard errors below the similarity values exhibited by cousins, and 1.52 standard errors below the 95% confidence interval surrounding all comparisons between full sibs. These two thresholds can be applied to pairs of wild wolves of unknown genealogy to estimate their general level of relatedness (see text). Standard errors in VNTR bandsharing are determined by the method of Lynch (1990). Note that this method generates standard errors which are independent of sample size and primarily dependent on the average number of VNTR fragments scored per individual. Statistics for the six $r$-value classes are as follows ($r$-value, number of comparisons made, range of $S$ values, average $S$ value $=S_x$, and standard error in $S_x$): $r=0$, 222, 405–641, 532, 71.4; $r=0.1875$, 12, 521–713, 640.8, 67.5; $r=0.25$, 5, 617–682, 649.6, 67.3; $r=0.375$, 1, 694, 694, 71.4; $r=0.5$, 21, 595–894, 753.9, 58.1; $r=0.75$, 9, 741–860, 787.2, 55.8. The standard error delineated in the graph is the largest of all classes (71.4); this is done to generate the most conservative thresholds for use in comparisons between wild wolves

demonstrating that the VNTR allelic diversity of the Forest Lake Colony is comparable to those of natural populations.

Using the relationships of similarity and $r$ in the Forest Lake colony we defined two levels of kinship. The first is defined by the lower bound of a 95% confidence interval about all comparisons of siblings. This bound falls at a value 108.5 points above the mean for unrelated individuals (Fig. 2). Using the maximum SE value of any of the classes ($SE=71.4$, Fig. 2), this value is 1.52 standard errors above the mean similarity of unrelated wolves. Thus all observed similarity values among wild-caught individuals that are more than 1.52 standard errors above the average value for unrelated individuals on that particular gel ($D>1.52$) represent pairs of wolves who are potentially related. Undoubtedly some spurious relationships will be deduced by using this threshold, as 2 of the 122 comparisons between unrelated individ-



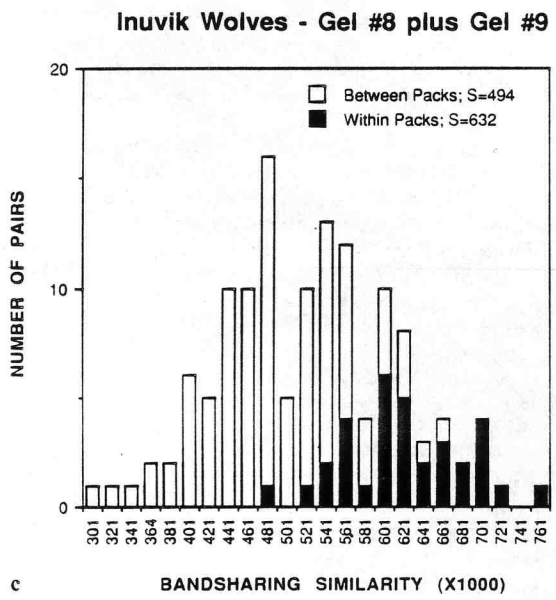




**Fig. 3a–c.** Sample histograms of actual VNTR similarity values within the three wild wolf pack clusters. **a** Sample values within the Minnesota pack cluster. **b** Sample values within the Denali pack cluster. **c** Sample values within the Inuvik pack cluster. For the Denali and Inuvik clusters, the histograms represent the combined bandsharing similarity values of two gels, each generated using different restriction enzymes on the same set of individuals (Table 3). For all three histograms, the $S$ values presented above the graphs are average values for all between- or within-pack comparisons (i.e. $S_x$ for those sets of comparisons)

uals within the Forest Lake colony generated similarities more than 1.52 standard errors above the average value. A more stringent level was defined as the lower bound of 1 SE from the mean of individuals related as siblings. This value is 2.29 standard errors above the mean similarity for unrelated wolves and delineates a class of wolves that includes only individuals related as closely as siblings or parent-offspring (Fig. 2).

*Within-pack comparisons*

The average similarity values among individuals within the same pack are always substantially higher than among wolves from different packs (Table 3). Histograms of similarity data clearly demonstrate the difference between within-pack and between-pack similarity values and show the comparisons that deviate from the expected trend (Fig. 3, see below). Although usual significance tests cannot be applied to pairwise similarity data because of the interdependence of similarity values (Dietz 1983), permutation tests for our data produced a significant division between the within- and among-pack average similarity values. Average differences in similarity greater than those found within actual packs are found in less than 5% of the 10,000 samplings of the data set of similarity values for each gel, except for the combined Minnesota gels (#2 plus #3, Table 3), which produce greater differences 21% of the time.

However, elements of similarity and mtDNA genotype distribution within our packs suggest a minimum of nine instances of more than two unrelated individuals ($D < 1.52$) existing within a pack (Table 4). In the Minnesota packs, male #75 of the Nip Creek pack has low

**Table 4.** Rejections of hypothesized pack structures

| Pack | Comparison | mtDNA genotype | VNTR similarity | *D* value |
|---|---|---|---|---|
| *Minnesota* | | | | |
| 1. Nip Creek | #75(M) vs. #197(F) | same | 424 | 0.52 |
| | #75(M) vs. #141(M) | same | 438 | 0.65 |
| | #75(M) vs. #151(M) | same | 333 | −0.32 |
| | #75(M) vs. #153(F) | same | 345 | −0.21 |
| 2. Isabella Lake | #103(M) vs. #97(F) | different | 426 | 0.59 |
| | #103(M) vs. #185(F) | different | 370 | −0.11 |
| | #103(M) vs. #193(F) | different | 333 | −0.23 |
| | #103(M) vs. #101(M) | same | no data | no data |
| 3. Emerald | #1(M) vs. #135(M) | same | 371 | −0.09 |
| *Denali* | | | | |
| 4. Birch | #369(F) vs. #371(F) | same | 566 | 1.02 |
| 5. McLeod Lake | #365(F) vs. #367(F) | same | 596 | 1.37 |
| *Inuvik* | | | | |
| 6. Wolverine | #90(F) vs. #15(F) | same | 500 | 0.15 |
| | #90(F) vs. #55(M) | same | 529 | 0.51 |
| | #90(F) vs. #56(F) | same | 581 | 0.23 |
| | #90(F) vs. #57(M) | same | 647 | 0.79 |
| 7. Island | #A(M) vs. #74(M) | same | 563 | 0.93 |
| 8. Rendezvous | #30(M) vs. #10(M) | same | 556 | 0.85 |
| | #30(M) vs. #70(M) | same | 571 | 0.14 |
| 9. Rat River | #95(M) vs. #96(M) | same | 483 | 0.58 |
| | #95(M) vs. #98(F) | same | 452 | 0.31 |

similarity values to four other apparent packmates, two of which are the same sex. This situation violates condition 1e (Table 1) for "standard" pack structure because only one comparison between different-sexed individuals should be so low if packs were composed only of unrelated parents and their offspring. The *D* value between the two males in the Emerald pack is less than zero, which also violates condition 1e in Table 1 for a pack structure composed of a breeding pair and their offspring.

Pack affiliations for the individuals assigned to the Isabella Lake pack are tentative, but assuming their accuracy, the genetic data indicate that this pack is not comprised only of a breeding pair and their offspring. Isabella Lake pack males #101 and #103 have a different mtDNA genotype than that three other female putative pack members, which violates condition 1c for pack structure (Table 1). Furthermore, #103 has low VNTR similarities to the pack females (Table 4), violating condition 1e as well.

Fewer intra-pack comparisons were possible for the Denali pack system, but deviations from the single breeding pair plus offspring pack system are observed as low similarity values among same-sexed individuals. In the Birch Creek and McLeod Lake packs, two members of the same sex (females in both cases) have low similarity values, indicating they are unlikely to be close relatives.

In the Inuvik pack system, for 4 of the 5 packs in which both the suspected breeding adults have been analyzed, the comparisons between the reproductive pairs produce *D* values that are either the lowest among all possible comparisons within the pack, or are within the unrelated range. In the Wolverine and Williams Lake packs, the suspected breeding males have a mtDNA genotype not found in other pack members; this situation is consistent with the "standard" pack structure. In the Island pack however, the putative breeding pair generates a *D* value of 2.22, which may indicate that a different pair is actually doing the breeding or that consanguineous mating is taking place. Four intra-pack values are inconsistent with packs being composed of a single reproducing pair and their offspring. In the Wolverine pack, the low similarity values of female #90 to other pack members suggest she is not a parent or sibling of other pack members (Table 4). Likewise, male wolf #30, the putative breeding male from the Rendezvous pack, generates low *D* values when compared to two other male packmates of the same mtDNA genotype. Such comparisons can also be found among the members of the Island and Rat River packs (Table 4).

Thus, while the majority of relationships within packs are concordant with the expectation that a dominant pair and their offspring will be the only members of a pack, our data provide clear examples of exceptions to this pack structure. For 27 packs, we used VNTR data to sample genetic relationships among two or more pack members, thereby allowing detection of deviations from the hypothesized standard pack structure. Nine of these packs contain genetic comparisons which suggest that a single pair of wolves and their offspring do not account for all of the individuals present in a pack.

*Between-pack comparisons*

Comparisons of similarity among individuals from different packs reveal several values that are greater than $D = 1.52$ or $D = 2.29$ standard errors from the mean of unrelated wolves. In the Minnesota pack cluster, seven closely-related ties ($D > 2.29$) connect packs (Fig. 4). Five of these involve connections between individuals who are not members of geographically adjoining packs (Figs. 1, 4). The remaining two are between individuals with the same mtDNA genotype from neighboring packs (Figs. 1, 4). However, one of these connections is between individuals in the Kawishiwi and Isabella Lake packs were pack affiliations were not certain. Thirteen additional connections among the Minnesota packs are indicated by the lower bound of relatedness ($D > 1.52$) which may include individuals only related approximately as first cousins ($0.5 > r > 0.1875$; Fig. 2) or unrelated individuals with high similarity values. Taken together, both classes of connections confirm that, in Minnesota, dispersal among packs of near relatives is a common event and contributes to the population structure of nearby wolf packs.

Connections at both levels are also apparent among the Denali packs, which are spread over a larger area (Figs. 1, 5). Six similarity values are more than 2.29 times the overall standard error above the unrelated individuals. Strong connections are suggested between the Stampede and East Fork packs, between the Chitsia and McKinley packs, between the Birch Creek and Little Bear packs, and between the Clearwater and McLeod Lake packs. Unlike in the Minnesota packs, the majority of these connections (5 of 6) are between packs that are either overlapping or share a common boundary. Moreover, behavioral observations indicate that both the Chitsia and Foraker packs are derivatives of the McKinley pack. Six more connections are suggested at the $D > 1.52$ level. Of these, four are between packs that share a territorial boundary.

The fewest connections among packs are apparent in the Inuvik pack cluster which, despite the high geo-

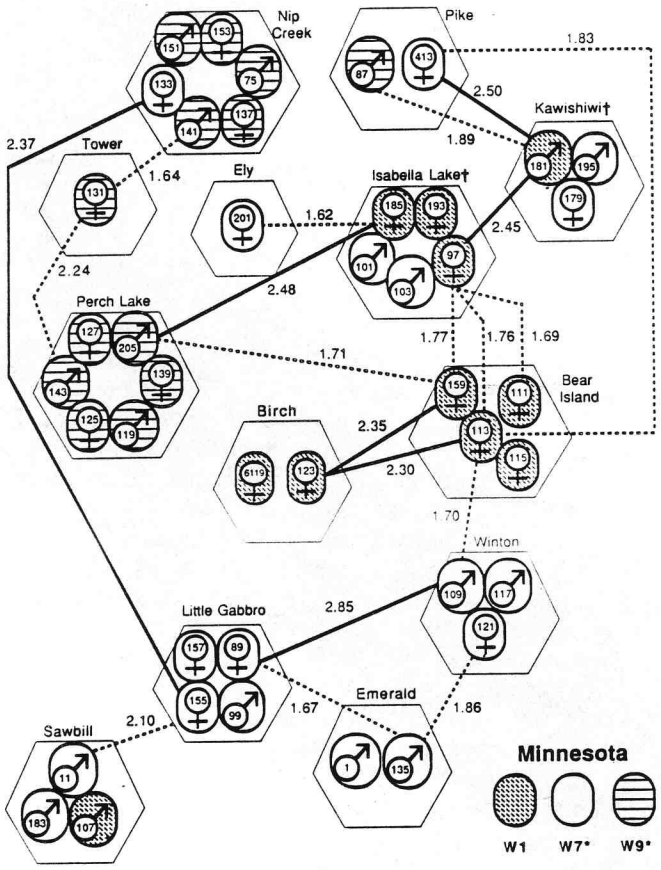



**Fig. 4.** Park compositions, mtDNA genotypes (W1, W7*, or W9*), and inter-pack connections suggested by high VNTR bandsharing values within the Minnesota pack cluster. The † indicates tentative pack assignments. MtDNA genotypes are indicated by the patterns of *shading* behind each wolf symbol; the *asterisks* by the W7 and W9 genotypes denote that these genotypes are derived from hybridization with coyotes, as per Lehman et al. (1991). The inter-pack connections are indicated by *lines* connecting individuals. *Solid lines* denote connections greater than 2.29 standard errors above the average value of unrelated individuals ($S_{unrelated}$) in the pack cluster. *Dashed lines* indicate connections between 1.52 and 2.29 standard errors above $S_{unrelated}$. The exact number of standard errors above $S_{unrelated}$ (the $D$ value) that each comparison generates is indicated next to each connection line. The placement of hexagons does not reflect the actual spatial relationships of the packs (see Fig. 1)

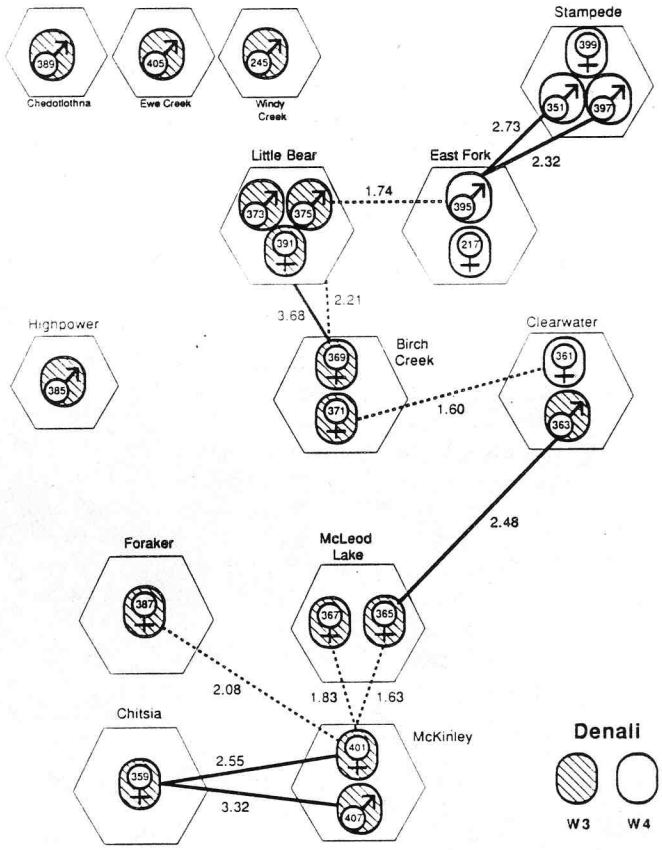



**Fig. 5.** Pack compositions, mtDNA genotypes (W3 or W4), and connections suggested by high inter-pack VNTR similarity values within the Denali (Alaska) pack cluster. Symbols and lines are as in Fig. 4. The Chedotlothna, Ewe Creek, and Windy Creek packs were subjected to a mtDNA analysis, but not to a VNTR analysis. The placement of hexagons does not reflect the actual spatial relationships of the packs (see Fig. 1)

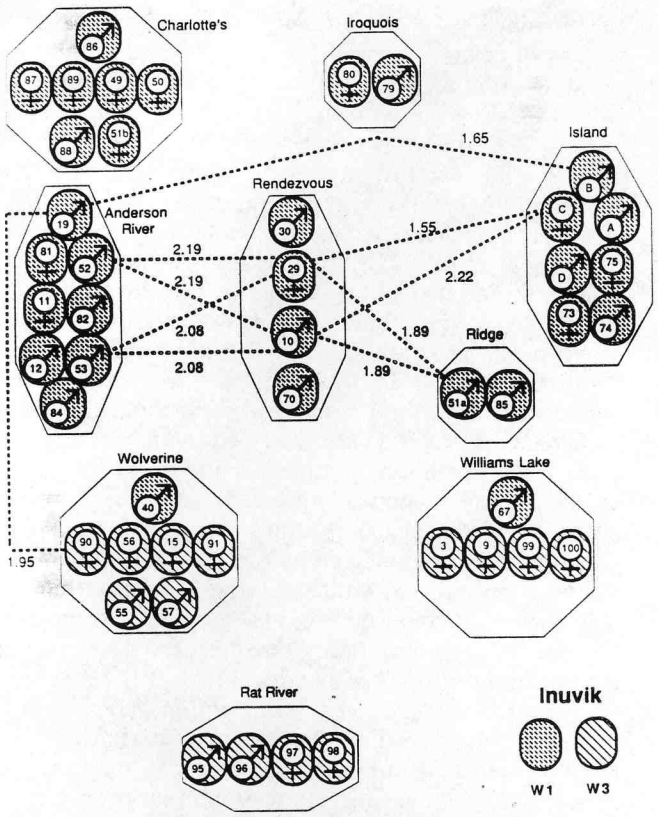



**Fig. 6.** Pack compositions, mtDNA genotypes (W1 or W3), and connections suggested by high inter-pack VNTR similarity values within the Inuvik (Northwest Territories, Canada) pack cluster. Symbols and lines as in Fig. 4. The placement of polygons does not reflect the actual spatial relationships of the packs (see Fig. 1)

graphical overlap of pack territories, is dispersed over the largest area (Table 1; Fig. 1). No connections are indicated at the $D > 2.29$ level, suggesting less frequent exchange of very close relatives among packs (Fig. 6). Ten connections are suggested by the lower bound ($D > 1.52$), and all of these connections are among packs that share parts of their territories.

Generally, fingerprint similarity among wolves from different packs in the three pack clusters reveals several connections among packs that suggest exchange of individuals related as closely as parent-offspring or siblings. In Minnesota and Alaska, many likely dispersal events are implied by strong connections between packs in the same cluster, while in the Northwest Territories, only weak inter-pack connections are observed.

### *Sex-biased dispersal*

Sex-dependent trends in similarity values were studied to detect a possible sex bias in dispersal and reproduction. Currently, there is not strong indication that one sex disperses farther than the other in gray wolves (Mech 1987; Gese and Mech 1991). For each gel, similarity values between members of the same sex were computed, excluding comparisons among pack members because they would tend to inflate the average and obscure inter-pack patterns (Table 5). In each pack system, the female-female average bandsharing similarities are higher than the male-male average similarities among packs (Table 5). The difference is most extreme in the Minnesota pack cluster, where pack boundaries exhibit the least geographical overlap.

**Table 5.** Analysis of sex-biased dispersal

| | Minnesota wolves | Denali wolves | Inuvik wolves |
|---|---|---|---|
| Amount of pack territorial overlap | Low | Moderate | High |
| Number of males in VNTR analysis | 18 | 8 | 24 |
| Number of females in VNTR analysis | 25 | 11 | 22 |
| Average interpack male-male similarities (sample size) | 388.8 (20) | 484.8 (33) | 489.2 (45) |
| Average interpack female-female similarities (sample size) | 442.2 (63) | 510.3 (43) | 521.4 (14) |
| Male-male interpack connections with $D > 1.52$ | 0 | 3 (all involve the same male) | 4 |
| Male-female interpack connections with $D > 1.52$ | 11 | 4 | 5 |
| Female-female interpack connections with $D > 1.52$ | 11 | 6 | 1 |
| $G$-test for sex-bias of connections | $G = 9.21$ ($P = 0.01$ with $df = 2$) | $G = 0.033$ ($P > 0.95$ with $df = 2$) | $G = 1.44$ ($P > 0.4$ with $df = 2$) |

For each pack cluster, the number of inter-pack similarity values with $D > 1.52$ were classified by the sexes of the pair (Table 5). In the Minnesota cluster, no connections were discovered between males of different packs, while half of the 22 connections were between females of different packs. This trend is not apparent in the other clusters. A $G$-test for goodness-of-fit (c.f. Sokal and Rohlf 1981) was performed on each cluster with the null hypothesis that connections between wolves of different packs would be formed independent of sex; only in the Minnesota cluster does the $G$-test suggest that the connections may have a significant sex bias (Table 5). However, it is important to note that only in the Minnesota cluster are the sample sizes large enough to allow a meaningful comparison between the test statistic and the chi-square distribution (Sokal and Rohlf 1981).

## Discussion

We have shown that genetic fingerprinting data can be used to deduce relationships in populations about which little genealogical information is known. Our approach uses the similarity values between individuals with different mtDNA genotypes to define a mean similarity of individuals unlikely to be closely related. We then establish standard error limits above this mean for two classes of relatedness. These error limits are based on the empirical relationship of similarity and relatedness in two groups of wolves of known genealogy. Our results establish three important behavioral characteristics of wolves. First, wolf packs are generally composed of individuals that are closely related (Mech 1970). Mean within-pack similarity values are significantly higher than values between packs for all three pack systems. However, several wolf packs deviate from the genetic structure expected if packs are composed only of an unrelated breeding pair and their offspring. Such exceptions were tentatively identified in one-third of the packs in which more than one individual was subjected to a combined VNTR-mtDNA analysis. Second, dispersal within pack clusters appears to occur frequently. Evidence of short-range dispersal becomes less apparent if pack territories overlap more extensively. Finally, dispersal may be sex-biased, as females in each cluster demonstrate higher values of relatedness than do males, and, in the Minnesota pack cluster, female-female genetic connections are significantly more frequent among packs than expected.

Behavioral studies of wild wolves have shown that pack members cooperate in the rearing, feeding, and protection of young (Murie 1944; Mech 1970). This degree of cooperative behavior can be explained by inclusive fitness theory if pack members are closely related (Hamilton 1964). Our data support this contention by demonstrating that packs are generally composed of closely-related wolves.

However, in several packs the genetic data are not consistent with all pack members being related except the breeding pair and support the existence of unrelated, non-breeding pack members (Rothman and Mech 1979; Van Ballenberghe 1983). In some packs, this result may be explained as a consequence of fusion of dispersing groups of related wolves (Mech and Nelson 1990). For example, among the wolves assigned to the Minnesota Isabella Lake pack, two males share the same genotype not found in the three other potential packmate females (Fig. 4). Fingerprint similarity values between male #103 and the three females are low ($D < 1$, Table 4), whereas the similarity between females #185 and #97 is high ($D > 1.52$). One scenario consistent with these results is that two sibling males from outside the pack system joined with female siblings originating from another pack, possibly the Kawishiwi, Bear Island, or Perch Lake packs to which there exist strong genetic connections with the Isabella Lake females. Alternatively, females #185 and #97 may be the only true Isabella Lake pack members sampled, and males #101 and #103, though genetically related, are not permanent members of the Isabella Lake pack. A series of inter-pack dispersal events such as these would tend to raise the between-pack $S_x$ values relative to the within-pack $S_x$ values; this could be the cause of the non-significant $P$ value for the permutation test performed on the combined Minnesota gels #2 and #3 (Table 3).

The existence of genetic connections ($D > 1.52$) between wolves from different packs indicates that short-range dispersal within pack clusters may be a frequent occurrence. Yet a genetic connection taken alone, in concert with no other data, does not differentiate between a dispersal event that led to the founding of a new pack or a dispersal from one pack to a second, pre-existing pack. Long-term field observations have documented few instances of pack-to-pack dispersal, and have suggested that the majority of wolves found new packs upon dispersal (Mech 1970; Gese and Mech 1991). For example, radiotelemetry studies performed in 1988–1990 indicate that only 3 of 12 wolves within the Denali region joined pre-existing packs upon dispersal from their native packs (L.D.M. and T.J.M., unpublished data). Similar studies on the Inuvik wolves show that approximately 20% dispersed to established packs within the study area (P.C., unpublished data).

Wolves sometimes form, or join, adjacent or nearby packs, and a minimum of about 25% of our packs exhibited genetic evidence of such occurrences (Figs. 1, 4–6). Such interchanges apparently occur despite the high level of aggression between wolves of neighboring packs, which sometimes results in significant mortality (Mech 1970, 1977; Van Ballenberghe et al. 1975). The frequency of dispersal events within the same cluster varies by region, with the most events observed in the Minnesota cluster and the fewest observed in the Inuvik cluster. In the Denali cluster, the genetic data describe a tendency for wolves to colonize areas close to their natal pack territories, as 4 of 5 strong inter-pack connections are between neighboring or overlapping packs (Figs. 1, 5). This tendency may be ascribed to pack fission (Mech 1966, 1986).

Compared to the other two pack clusters, wolves in the Inuvik region exhibit fewer inter-pack genetic connections, none of which is strong ($D > 2.29$). Two explanations, which are not mutually exclusive, are apparent

for this situation. First, not all packs in the area spanned by the vast Inuvik study area were sampled. In contrast to the Minnesota and Denali clusters, where we have sampled 80% or more of the packs in the region, we conservatively estimate that fewer than half of the existing Inuvik packs were included in our study. Therefore, genetic connections between packs may have been missed as a consequence of insufficient sampling. Second, wolves in this area may be dispersing farther. Inuvik wolves often wander great distances in pursuit of caribou herds and do not orient around familiar landmarks as frequently as Minnesota wolves (Clarkson and Liepens 1989). Their territorial ranges are extensive, often surpassing the areas of the Minnesota and Denali clusters, and these ranges overlap almost entirely when viewed over a year (Fig. 1). Consequently these wolves may disperse outside of the cluster area in order to take up residence in a region not encompassed by their natal territories.

The apparent female bias in among-pack similarity values was not expected given prior observations of wolf dispersal, which demonstrated that both male and female wolves travel long distances (Mech 1987; Gese and Mech 1991). The sex bias could reflect differential mortality of males in wolf packs during dispersal events, differential reproductive success of the two sexes, or males more frequently dispersing long distances. The third of these three alternatives is supported by the weakening of the sex-bias trend as pack cluster range increases; the Minnesota cluster may span an area small enough for a trend to be apparent, while the Inuvik cluster may span an area too large to reveal a sex bias in dispersal.

In regards to the benefits of our molecular-genetic approach, it does not require a detailed knowledge of the source population and is accomplished with less expense and laboratory analysis than if all individuals needed to be sampled and compared. Moreover, each electrophoretic gel can be analyzed independently, and with appropriate controls, results can be compared from analyses done at different times and in various laboratories. However, the approach is limited in that individuals with different mtDNA genotypes must be included on the same gel so that the mean similarity among individuals unlikely to be related can be defined. In addition, it requires setting limits of relatedness based on analyses of individuals of known genealogy. The approach is best applied to monogamous species.

The deficiencies of our method are that it provides only a limited number of comparisons among individuals unless multiple electrophoretic gels are performed and analyzed. Thus we are unlikely to have found all possible links between individuals within or between packs. Our approach assumes that the relative distribution of similarity values in unrelated and related individuals is similar in different populations (Fig. 3). This clearly may not be realized in populations that are small and highly isolated or that have suffered a recent population bottleneck (Gilbert et al. 1990; Packer et al. 1991). However, in large and stable outbred populations, such as in the gray wolf, this bias is expected to be less important.

*Acknowledgements.* We would like to thank the many collaborators who provided gray wolf tissue specimens, especially J. Burch, P. Kinnis, and T. Kreeger. In addition we thank A. Eisenhawer, P. Fraser, P.J.P. Gogan, K. Hansen, B. Jacobson, I. Jacobson, I. Liepins, and N.R. Sturm for technical assistance and advice. We also appreciate the helpful comments on the manuscript made by E. Geffen, R. Gibson, D. Girman, M. Gomper, F. Hertel, S. Jenks, G. Roemer and B. Van Valkenburgh. This work was supported in part by a U.S.P.H.S. National Service Award GM-07104 to N.L., a U.S. National Park Service agreement with R.K.W., a National Science Foundation grant to R.K.W. (BSR 9020282), as well as by the U.S. Fish and Wildlife Service, the U.S.D.A. North Central Forest Experiment Station, the U.S. National Park Service, the Department of Renewable Resources of the Government of the Northwest Territories, and the Inuvialuit Game Council and Wildlife Management Advisory Council of the Northwest Territories.

## References

Burke T (1989) DNA fingerprinting and other methods for the study of mating success. Trends Ecol Evol 4:139–144

Clarkson P, Liepens I (1989) Inuvialuit wildlife studies: western arctic wolf research project progress report, 1988–1989. Northwest Territories Wildlife Management Advisory Council Technical Report No. 7

Dietz EJ (1983) Permutation tests for association between two distance matrices. Syst Zool 32:21–26

Fritts SH, Mech LD (1981) Dynamics, movements, and feeding ecology of a newly protected wolf population in northwestern Minnesota. Wildl Monogr 80:1–79

Fuller TK (1989) Population dynamics of wolves in north-central Minnesota. Wildl Monogr 105:1–41

Gese EM, Mech LD (1991) Dispersal of wolves (*Canis lupus*) in northeastern Minnesota, 1969–89. Can J Zool 69:2946–2955

Gilbert DA, Lehman N, O'Brien SJ, Wayne RK (1990) Genetic fingerprinting reflects population differentiation in the California Channel Island Fox. Nature 344:764–767

Hamilton WD (1964) The genetical evolution of social behavior. I and II. J Theor Biol 7:1–52

Harrington FH, Ryon J, Fentress JC (1982) Monogamy in wolves: a review of the evidence. In: Harrington FH, Paquet PC (eds) Wolves in the world. Noyes Publications, Park Ridge, NJ, pp 209–222

Jeffreys AJ, Wilson V, Thein SL (1985) Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73

Kleiman DG, Eisenberg J (1973) Comparison of canid and felid social systems from an evolutionary perspective. Anim Behav 21:637–659

Lehman N, Eisenhawer A, Hansen K, Mech LD, Peterson RO, Gogan PJP, Wayne RK (1991) Introgression of coyote mitochondrial DNA into sympatric North American gray wolf populations. Evolution 45:104–119

Lynch M (1988) Estimation of relatedness by DNA fingerprinting. Mol Biol Evol 5(5):584–599

Lynch M (1990) The similarity index and DNA fingerprinting. Mol Biol Evol 7(5):478–484

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, New York

Macdonald DW, Moehlman PD (1982) Cooperation, altruism, and restraint in the reproduction of carnivores. In: Bateson PPG, Klopfer P (eds) Perspectives in Ethology. Plenum Press, New York, pp 433–467

Mech LD (1966) The wolves of Isle Royale. U.S. Government Printing Office, Washington D.C.

Mech LD (1970) The Wolf: Ecology and behavior of an endangered species. Doubleday, New York

Mech LD (1973) Wolf numbers in the Superior National Forest of Minnesota. U.S.D.A. Forest Service Research Paper NC-97:1–10

Mech LD (1977) Productivity, mortality and population trends of wolves in northeastern Minnesota. J Mammal 58:559–574

Mech LD (1986) Wolf population in the central Superior National Forest, 1967–1985. U.S.D.A. North Central Forest Experiment Station Research Paper NC-270:1–6

Mech LD (1987) Age, season, distance, direction, and social aspects of wolf dispersal from a Minnesota pack. In: Chepko-Sade BD, Halpin ZT (eds) Mammalian dispersal patterns. University of Chicago Press, Chicago, pp 55–74

Mech LD, Nelson ME (1990) Non-family wolfpacks. Can Field Nat 104:482–483

Murie A (1944) The wolves of Mt. McKinley. US Natl Park Serv, Fauna Ser 5:1–238

Packard JM, Mech LD, Seal US (1983) Social influences on reproduction in wolves. In: Carbyn LN (ed) Wolves in Canada and Alaska. Canadian Wildlife Service Report Series No. 45, pp 78–85

Packer C, Gilbert DA, Pusey AE, O'Brien SJ (1991) A molecular genetic analysis of kinship and cooperation in African lions. Nature 351:562–565

Peterson RO (1977) Wolf ecology and prey relationships on Isle Royale. US Natl Park Serv Sci Monogr 11:1–210

Rothman RJ, Mech LD (1979) Scent marking in lone wolves and newly formed pairs. Anim Behav 27:750–760

Sokal RR, Rohlf FJ (1981) Biometry, 2 edn. W.H. Freeman, New York

Van Ballenberghe V (1983) Extraterritorial movements and dispersal of wolves in south central Alaska. J Mammal 64:168–171

Van Ballenberghe V, Erikson AW, Byman D (1975) Ecology of the timber wolf in northeastern Minnesota. Wildl Monogr 43:1–43

Wayne RK, Gilbert DA, Lehman N, Hansen K, Eisenhawer A, Girman D, Peterson RO, Mech LD, Gogan PJP, Seal US, Krumenaker RJ (1991) Conservation genetics of the endangered Isle Royale gray wolf. Conserv Biol 5(1):41–51

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147–149