# Inbreeding and relatedness in Scandinavian grey wolves *Canis lupus*

HANS ELLEGREN

*Department of Evolutionary Biology, Uppsala University, Uppsala, Sweden.*

Management of small and threatened populations may require detailed knowledge about the genetic status of individuals and the genetic relatedness between individuals. I show here that individual heterozygosity at a set of 29 microsatellite loci correlates closely to the degree of inbreeding in a captive grey wolf population. Microsatellite allele sharing similarly correlates closely to known relatedness between pairs of individuals. Genotyping the same markers in a small (60–70 individuals) natural population of grey wolves in Sweden, low individual heterozygosities and high values of allele sharing between some animals were found. Since inbreeding depression has been documented in a captive grey wolf population of Scandinavian origin, the results point out an additional risk for the small Swedish wild population.

*Hans Ellegren, Department of Evolutionary Biology, Uppsala University, Norbyvägen 18D, SE-752 36 Uppsala, Sweden.*
E-mail: hans.ellegren@evolution.uu.se

Analyses of endangered populations may require detailed knowledge about genetic variability and genetic relatedness among individuals. Such information can also be of significant importance for the proper design of conservation programs. This includes, for instance, avoidance of matings between close relatives in captive stocks in the absence of pedigree data, estimates of minimum viable wild population sizes, and considerations of the need for translocation (HEDRICK and MILLER 1992; EBENHARD 1995; GIRMAN et al. 1997). Assessment of genetic variability in populations has been greatly facilitated by the advent of high-resolution, molecular genetic techniques for population genetic studies. Recently, PCR-based microsatellites have proved to be ideal genetic markers for population studies, particularly because of locus-specificity, discrete allele sizes, hypervariability and minute amounts of sample required for analysis (reviewed by BRUFORD and WAYNE 1993; QUELLER et al. 1993; JARNE and LAGODA 1996). Microsatellites have in several cases been used to uncover low levels of genetic variability in bottlenecked populations (GOTTELLI et al. 1994; TAYLOR et al. 1994; HOULDEN et al. 1996; ELLEGREN et al. 1996), but studies quantitatively addressing how individual heterozygosity at microsatellite loci relates to the loss of genomic variability due to inbreeding in small populations are rare. Also, while it has been shown that microsatellite data efficiently can reconstruct hierarchical genetic relationships among individuals (BOWCOCK et al. 1994; BLOUIN et al. 1996; ELLEGREN et al. 1996), there have been few attempts to calibrate allelic similarity at microsatellite loci to known degree of relatedness between inbred pairs of individuals (cf. BLOUIN et al. 1996).

Inferring the increase in homozygosity due to inbreeding and inferring relatedness between individuals in a small, threatened population may be critical for its management. This probably applies to the current situation for the Swedish grey wolf *Canis lupus* population. Following lengthy persecution, the grey wolf became practically extinct from the Scandinavian peninsula in the 1970s, a situation similar to what has been the case in many other parts of the world where the species formerly was wide-spread and common (MECH 1995). In the early 1980s, however, grey wolves were again sighted in central Sweden and the population has since increased in size to some 60–70 individuals. Since there has been no regular occurrence of grey wolves in northern Sweden in recent years, gene flow between the Swedish population and wolves from the large Russian population, through Finland, might not have taken place. The current Swedish population is therefore likely to have been isolated since it was founded. This is supported by the fact that from a sample of 13 Swedish wolves collected after the population reappeared, only a single mitochondrial DNA haplotype was found (ELLEGREN et al. 1996).

With few founders and no gene flow, the Swedish grey wolf population may have experienced a loss of genetic variability due to founder effects and genetic drift (NEI et al. 1975; ALLENDORF 1986). Genotyping of 12 microsatellite loci indicated moderate levels of genetic variability and a loss of genetic diversity over time, i.e. individual heterozygosity was found to be lower among animals born recently (ELLEGREN et al. 1996). Parallel to this, it is possible that inbreeding takes place in this population. This could at least potentially be harmful for the long-term survival of

wolves in Sweden since severe inbreeding depression has been recorded among captive wolves originally taken from the Scandinavian population (LAIKRE and RYMAN 1991). Such problems may be of particular importance in a situation where the low population size also makes the population sensitive to demographic stochasticity.

In this study, I have genotyped 29 microsatellites in a captive and inbred population of wolves, and used these data to establish relationships between inbreeding and individual heterozygosity, and between relatedness and allelic similarity. In both cases, microsatellite data correlate closely to the measures of genetic relatedness. By genotyping the same markers in animals from the wild Swedish population, the established relationships have been used to make inferences on the genetic status of wild wolves.

## MATERIALS AND METHODS

### Samples

Twenty-nine captive wolves from Swedish zoos were analysed. Samples either constituted tissue or serum which had been stored at $-20°C$ for 2–15 years. Originally founded by two wild-caught Swedish and Finnish sib-pairs, the Swedish zoo population has suffered from several generations of intense inbreeding, in recent years to some extent neutralised by the introduction of a few Russian (one sib-pair) and Estonian (three siblings) animals in the breeding programme. The structure of the pedigree involving the 29 captive wolves analysed in this study is shown in Fig. 1. Among these 29 wolves were the 21 animals analysed by ELLEGREN et al. (1996). Coefficients of inbreeding f and relatedness r (Wright 1922) were calculated with the program Wildlife (Per-Erik Sundgren pers. comm.). Thirteen wild wolves had either been road killed or had been legally or illegally shot during 1984–1994; this sample constitutes all dead wild wolves known by national authorities during this period. These animals corresponds to wolves V1-V13 in ELLEGREN et al. (1996), where sampling localities also are indicated. Tissue samples were available for these animals.

### DNA analysis

DNA preparation and microsatellite genotyping procedures were essentially as described in ELLEGREN et al. (1996). Briefly, 100 ng of genomic wolf DNA was used in 10 µl PCR amplifications with 2 pmol of each primer (one end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$), standard PCR buffer containing 1.5 mM $MgCl_2$ and 0.25 U *Taq* polymerase (Dynazyme). Cycling conditions were an initial denaturation of 94°C for 3 min fol-

lowed by 30 cycles of 94°C for 30 s, 50–65°C for 30 s and 72°C for 45 s. Annealing temperatures were as in original marker reports. PCR products were separated in 6% polyacrylamide gels and detected by autoradiography. A total of 29 canine microsatellites were used: AHT101 and 106 (HOLMES et al. 1993), AHT124, 125 and 126 (HOLMES et al. 1994), AHT103, 119, 121, 133, 136 and 138 (HOLMES et al. 1995), AHTk39 (N.G. Holmes, unpublished), VIAS-D10 (PRIMMER and MATTHEWS 1993), vWF (SHIBUYA et al. 1994), 109, 123, 172, 173, 204, 213, 225, 250 and 377 (OSTRANDER et al. 1993), and 2001, 2010, 2054, 2088, 2137 and 2159 (FRANCISCO et al. 1996).

### Data analysis

Polymorphism at microsatellite loci was measured as allelic diversity (number of alleles per locus) A, observed heterozygosity $H_o$ and expected heterozygosity $H_e$. Deviations from Hardy-Weinberg expectations were analysed by chi-square tests after pooling of rare alleles, yielding three classes of genotypes; homozygotes for the most common allele, heterozygotes for the most common allele, and others. Significance levels were corrected for simultaneous tests. An unbi-
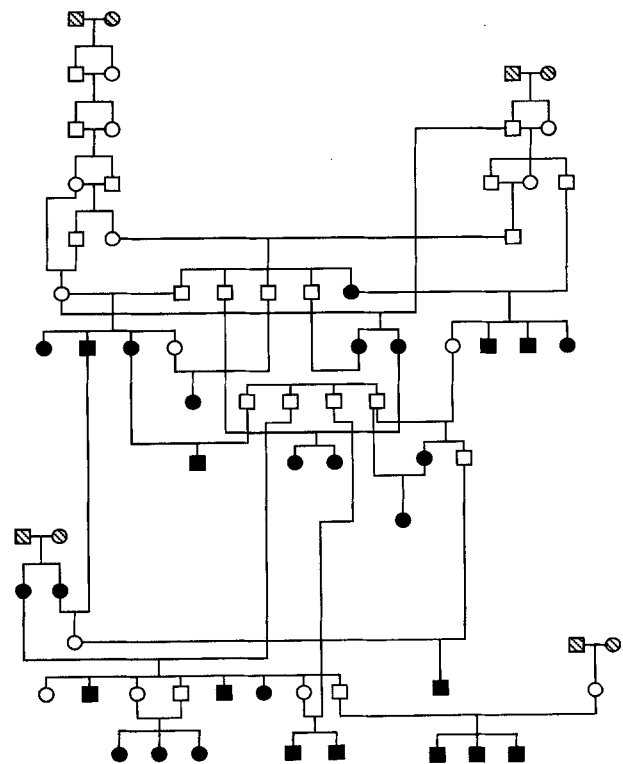


**Fig. 1.** Pedigree of the captive Swedish wolf population. Only the parts of the pedigree leading to the animals analysed in this study (filled symbols) are shown. Founders are indicated by shaded symbols.
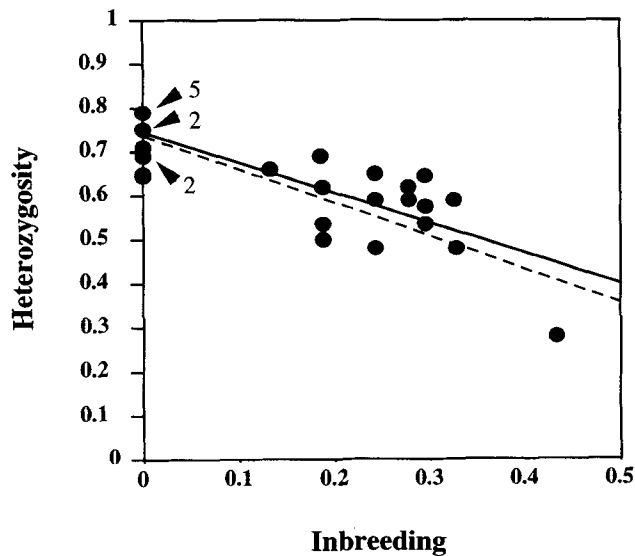
**Fig. 2.** The relationship between individual heterozygosity (H$_i$) at microsatellite loci and inbreeding (f) in a captive wolf population. The straight line represents the regression of H$_i$ on f, the dashed line is the expected relationship between H$_i$ and f, assuming an H$_i$ of 0.75 among unrelated individuals.

ased estimate of true genetic relationships can be derived if outbred population allele frequencies are known but since this was not the case in this study, The statistic r$_{xy}$ by QUELLER and GOODNIGHT (1989) could not be applied. As a measure of allele sharing I used the average proportion of shared alleles M$_{xy}$ (Ps in BOWCOCK et al. 1994). M$_{xy}$ is simply calculated as the total number of identical alleles in a pair of individuals, 0, 1 or 2 per locus, divided by twice the number of loci analysed. BLOUIN et al. (1996) found the distribution of M$_{xy}$ among related mice to be similar that of r$_{xy}$. M$_{xy}$ was calculated using the program MICROSAT (GOLDSTEIN et al. 1995).

## RESULTS

### Heterozygosity in relation to inbreeding

Microsatellite variability within a sample of 29 captive wolves which had experienced various degrees of inbreeding was moderate with an average A of 2.9 (SD = 0.9; range 1–4) and a mean H$_e$ of 0.51 (SD = 0.20; 0–0.76) among the 29 loci investigated. The fact that a large number of markers was genotyped implies that an idea of the degree of genomic variability in individual wolves can be derived from the proportion of loci present in heterozygote state (individual heterozygosity, H$_i$). That this measure correlates closely with overall genome variability is evident from a comparison of H$_i$ and f in captive wolves (Fig. 2; r$^2$ = 0.67, P = 0.0001). While unrelated wolves

showed heterozygosities of 0.7–0.8, most animals with f = 0.2–0.3 had heterozygosities of 0.5–0.6. The most inbred (f = 0.42) animal had H$_i$ = 0.28. The slope of the regression "H$_i$ = 0.74 − 0.68 × F" does not differ significantly from the expected loss of heterozygosity due to inbreeding (1 − [f × H]), using the mean H$_0$ among unrelated wolves (0.75) for expectations (t = 0.73, P > 0.1).

Samples from 13 wild wolves gathered after the reappearance in Sweden in the beginning of the 1980s were available. Genotyping the same set of microsatellites for the 13 wild wolves revealed genetic diversity similar to the captive population, i.e. mean A = 3.1 (SD = 1.0; range 1–5) and mean H$_e$ = 0.52 (SD 0.13, 0 0.71). No locus showed significant deviation from Hardy-Weinberg expectations (data not shown). Mean H$_i$ among wild wolves was 0.54 (SD = 0.17). From the distributions of individual heterozygosities among wild and among captive animals, the genetic variability of about half of the wild wolves was as low as that among inbred captive wolves (Fig. 3). One wild animal had an even lower heterozygosity (H$_i$ = 0.25) than the most inbred captive animal.

### Microsatellite allele sharing and relatedness

The access to pedigree data for the captive wolves also allows an analysis of how microsatellite allele sharing for pairs of individuals vary over a broad range of genetic relatednesses. With 29 animals, 406 dyads can be formed (the number of dyads from a set of n individuals is (n$^2$ − n)/2), and for these r varied between 0 and 1.04, and M$_{xy}$ between 0.17 and 0.96. There was a very good agreement between relatedness and allele sharing (Fig. 4; r$^2$ = 0.72, P < 0.0001), indicating that this set of microsatellites should be able to efficiently identify related individuals in a sample of animals of unknown genealogy.
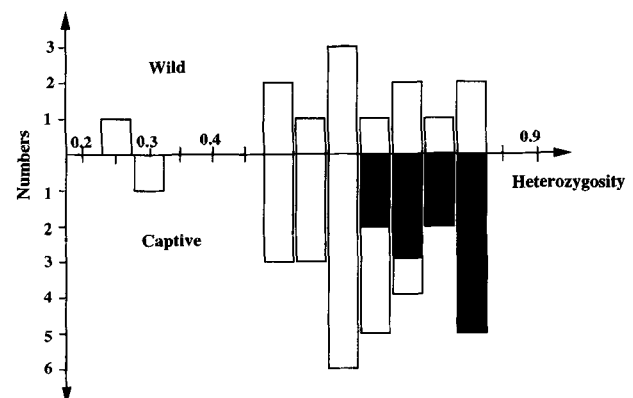


**Fig. 3.** Distribution of individual heterozygosities (H$_i$) among wild (above) and captive (below) wolves. For captive wolves, filled bars represent data for non-inbred animals and open bars data for inbred animals (f > 0; the lowest f value was 0.13).

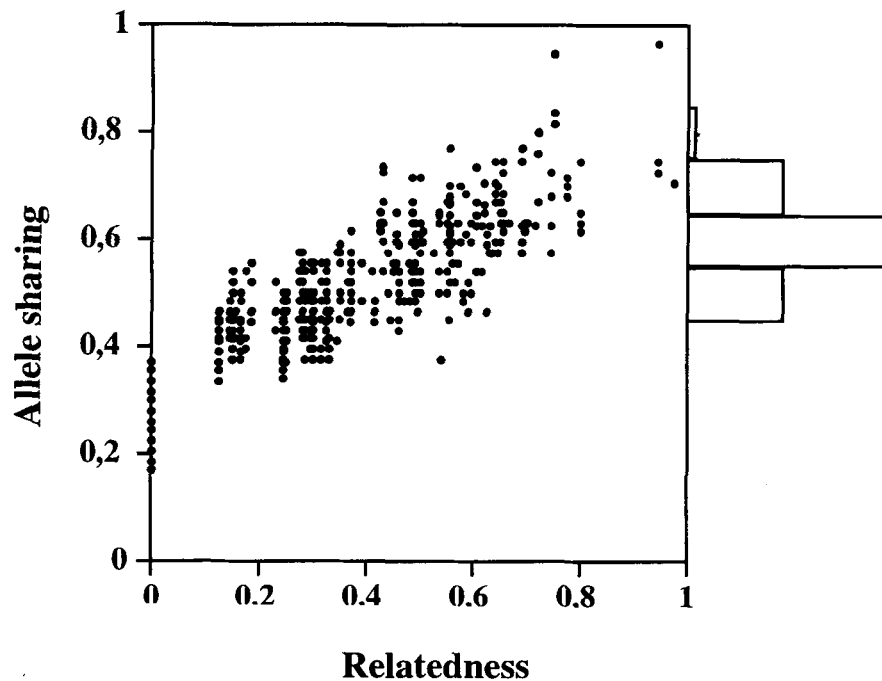**Fig. 4.** A composite figure showing the relationship between allele sharing ($M_{xy}$) and relatedness (r) among captive wolves, and the distribution of values of allele sharing among wild wolves (to the right of the diagram).

Allele sharing among wild wolves ranged from 0.45 to 0.75 which suggested that many of these animals are related, in some cases to an extent corresponding to that among inbred animals (Fig. 4). Twenty dyads of wild wolves had allele sharing of 0.65 or higher which, by interpolation, would imply a relatedness of at least 0.5.

## DISCUSSION

This study demonstrates that individual heterozygosity at microsatellite loci from a small grey wolf population correlates closely to the degree of inbreeding and that microsatellite allele sharing similarly correlates closely to the relatedness between pairs of individuals. These relationships are not surprising but none the less important to establish for evaluating the usefulness and the application of microsatellites in genetic studies of endangered populations. Heterozygosity and allele sharing should be expected to respond linearly to inbreeding and relatedness, respectively, as was also the case (in contrast to band sharing in multi-locus DNA fingerprinting; KUHNLEIN et al. 1990). Calibrations presented herein could thus prove useful in genetic studies of the many endangered grey wolf populations over the world (cf. WAYNE et al. 1991; GOTTELLI et al. 1994; ROY et al. 1994; FORBES and BOYD 1996; GARCíA-MORENO et al. 1996; HEDRICK et al. 1997).

A critical parameter in this type of analysis is the number of markers used. The capability of a set of microsatellites to correctly resolve true relatedness increases more than linearly with an increasing number of markers (TAKEZAKI and NEI 1996). The present study was based on 29 markers which may be considered as an appropriate number in this respect (TAKEZAKI and NEI 1996). For most wildlife species for which microsatellites so far have been developed, the number of available markers is generally much less. Another factor that will affect the relationships between microsatellite variability and relatedness is the intrinsic variation in polymorphism content for repeats of different length, polymorphism generally being higher for longer repeats (WEBER 1990). However, heterozygosity appears to reach a plateau level for loci with a mean allelic length of 15–20 repeat units, and almost all markers used in this study contained at least 15 repeat units in the cloned allele.

Microsatellite data suggest that at least some grey wolves from the wild Swedish population are related to an extent that mirrors what is seen among known inbred animals. Increased homozygosity because of inbreeding may not per se be threatening to small populations if, for instance, the gene pool has been purged of deleterious alleles (MEROLA 1994; BALLOU 1997; LACY and BALLOU 1998). Indeed there are examples of natural mammalian populations which appear to do well despite harbouring low levels of

genetic diversity (SJÖGREN 1991; ELLEGREN et al. 1993), and the genetic load can obviously vary between populations (RALLS et al. 1988). However, as mentioned, inbreeding depression has been documented among captive Scandinavian wolves (LAIKRE and RYMAN 1991; LAIKRE et al. 1993), and has also been suggested for other wolf populations (WAYNE et al. 1991; GARCÍA-MORENO et al. 1996).

It is not yet known if the wild Swedish grey wolf population actually suffers from any possible detrimental effects of inbreeding. The expression of inbreeding depression may in itself be environmentally dependent, but one would in that case expect an increased severity under natural conditions than in captivity (JIMÉNEZ et al. 1994). Coupled with the needs for genetic variability in the resistance against epizootics (O'BRIEN and EVERMANN 1988) and, in a longer perspective, in evolutionary adaptation (FRANKEL and SOULÉ 1981), it seems important that the Scandinavian grey wolf population can establish gene flow with the large Russian population. The genetic contribution of a few immigrants may significantly counteract the negative effects of inbreeding (SPIELMAN and FRANKHAM 1992).

## ACKNOWLEDGEMENTS

## REFERENCES

Allendorf FW, (1986). Genetic drift and the loss of alleles versus heterozygosity. Zoo Biol. 5: 181–190.

Ballou JD, (1997). Ancestral inbreeding only minimally affects inbreeding depression in mammalian populations. J. Hered. 88: 169–178.

Blouin MS, Parsons M, Lacaille V and Lotz S, (1996). Use of microsatellites to classify individuals by relatedness. Mol. Ecol. 5: 393–401.

Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR and Cavalli-Sforza LL, (1994). High resolution of human evolutionary trees with polymorphic microsatellites. Nature 368: 455–457.

Bruford MW and Wayne RK, (1993). Microsatellites and their application to population genetic studies. Current Opinion in Genetics and Development 3: 939–943.

Ebenhard T, (1995). Conservation breeding as a tool for saving animal species from extinction. Trends in Ecology and Evolution 10: 438–443.

Ellegren H, Hartmann G, Johansson M and Andersson L, (1993). Major histocompatibility complex monomorphism and low levels of DNA fingerprinting variability in a reintroduced and rapidly expanding population of beavers. Proc. Natl. Acad. Sci. USA 90: 8150–8153.

Ellegren H, Savolainen P and Rosén B, (1996). The genetical history of an isolated population of the endangered grey wolf Canis lupus: a study of nuclear and mitochondrial polymorphisms. Philos. Trans. R. Soc. Lond. B 351: 1661–1669.

Forbes SH and Boyd DK, (1996). Genetic variation of naturally colonizing wolves in the central Rocky Mountains. Conserv. Biol. 10: 1082–1090.

Francisco LV, Langston AA, Mellersh CS, Neal CL and Ostrander EA, (1996). A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. Mamm. Genome 7: 359–362.

Frankel OH and Soulé ME, (1981). Conservation and Evolution. Cambridge University Press, Cambridge.

García-Moreno J, Matocq MD, Roy MS, Geffen E and Wayne RK, (1996). Relationships and genetic purity of the endangered Mexican wolf based on analysis of microsatellite loci. Conserv. Biol. 10: 376–389.

Girman DJ, Mills MGL, Geffen E and Wayne RK, (1997). A molecular genetic analysis of social structure, dispersal, and interpack relationships of the African wild dog (Lycaon pictus). Behav. Ecol. Sociobiol. 40: 187–198.

Goldstein DB, Linares AR, Cavalli-Sforza LL and Feldman MW, (1995). An evaluation of genetic distances for use with microsatellite loci. Genetics 139: 463–471.

Gottelli D, Sillero-Zubiri C, Applebaum GD, Roy MS, Girman DJ, García-Moreno J, Ostrander E and Wayne RK, (1994). Molecular genetics of the most endangered canid: the Ethiopian wolf Canis simensis. Mol. Ecol. 3: 301–312.

Hedrick PW and Miller PS, (1992) Conservation genetics: techniques and fundamentals. Ecological Appl. 2: 30–46.

Hedrick PW, Miller PS, Geffen E and Wayne RK, (1997). Genetic evaluation of the three captive Mexican wolf lineages. Zoo Biol. 16: 47–69.

Holmes NG, Mellersh CS, Humphreys SJ, Binns MM, Holliman A, Curtis R and Sampson J, (1993). Isolation and characterization of microsatellites from the canine genome. Animal Genetics 24: 289–292.

Holmes NG, Strange NJ, Binns MM, Mellersh CS and Sampson J, (1994). Three polymorphic canine microsatellites. Anim. Genet. 25: 200.

Holmes NG, Dickens HF, Parker HL, Binns MM, Mellersh CS and Sampson J, (1995) Eighteen canine microsatellites. Anim. Genet. 26: 132–133.

Houlden BA, England PR, Taylor AC, Greville WD and Sherwin WB, (1996). Low genetic variability of the koala Phascolarctos cinereus in south-eastern Australia following a severe population bottleneck. Mol. Ecol. 5: 269–281.

Jarne P and Lagoda PJL, (1996). Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution 11: 424–429.

Jiménez JA, Hughes KA, Alaks G, Graham L and Lacy RC, (1994). An experimental study of inbreeding depression in a natural habitat. Science 266: 271–273.

Kuhnlein U, Zadworny D, Dawe Y, Fairfull RW and Gavora JS, (1990). Assessment of inbreeding by DNA fingerprinting: development of a calibration curve using defined strains of chicken. Genetics 125: 161–165.

Lacy RC and Ballou JD, (1998). Effectiveness of selection in reducing the genetic load in populations of Peromyscus polionotus during generations of inbreeding. Evolution 52: 900–909.

Laikre L and Ryman N, (1991). Inbreeding depression in a captive wolf (Cants lupus) population. Conserv. Biol. 5: 33–40.

Laikre L, Ryman N and Thompson EA, (1993). Hereditary blindness in a captive wolf: (Canis lupus: population: frequency reduction of a deleterious allele in relation to gene conservation. Conserv, Biol. 7: 592–601.

Mech LD, (1995). The challenge and opportunity of recovering wolf populations. Conserv. Biol. 9: 270–278.

Merola M, (1994). A reassessment of homozygosity and the case for inbreeding depression in the Cheetah, Acionyx jubatus: Implications for conservation. Conserv. Biol, 8: 961–971.

Nei M, Maruyama T and Chakraborty R, (1975). The bottleneck effect and genetic variability in populations. Evolution 29: 1–10.

O'Brien SJ and Evermann JF, (1988). Interactive influence of infectious disease and genetic diversity of natural populations. Trends Ecol. Evol. 3: 254–259.

Ostrander EA, Sprague GF, Jr and Rine J, (1993). Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in the dog. Genomics 16: 207–213.

Primmer CR and Matthews ME, (1993). Canine tetranucleotide repeat polymorphism at the VIAS-D10 locus. Anim. Genet. 24: 332.

Queller DC, Goodnight KF, (1989). Estimating relatedness using genetic markers. Evolution 43: 258–275.

Queller DC, Strassmann JE and Hughes CR, (1993). Microsatellites and kinship. Trends Ecol. Evol. 8: 285–288.

Ralls K, Ballov JD, and Templeton A, (1998). Estimates of lethal equivalents and the cost of inbreeding in mammals. Conserv. Biol. 2: 185–193.

Roy MS, Geffen E, Smith D, Ostrander EA and Wayne RK, (1994). Patterns of differentiation and hybridization in North American wolflike canids, revealed by analysis of microsatellite loci. Mol. Biol. Evol. 11: 553–570.

Shibuya H, Collins BK, Huang THM and Johnson GS, (1994). A polymorphic $(AGGAAT)_n$ tandem repeat in an intron of the canine von Willebrand factor gene. Anim. Genet. 25: 122.

Sjögren P, (1991). Genetic variation in relation to demography of peripheral pool frog populations (Rana lessonae). Evol. Ecol 5: 248–271.

Spielman D and Frankham R, (1992). Modeling problems in conservation genetics using captive Drosophila populations: Improvement of reproductive fitness due to immigration of one individual into small partially inbred populations. Zoo Biol. 11: 343–351.

Takezaki N and Nei M, (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144: 389–399.

Taylor AC, Sherwin WB and Wayne RK, (1994). Genetic variation of microsatellite loci in a bottleneck species: the northern hairy-nosed wombat Lasiorhinus krefftii. Mol. Ecol. 3: 277–290.

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

Weber JL, (1990). Informativeness of human $(dC-dA)_n \cdot (dG-dT)_n$ polymorphisms. Genomics 7: 524–530.

Wright S, (1922). Coefficients of inbreeding and relationship. Am. Nat. 56: 330–338.