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Genetic diversity, population structure, effective population size and demographic history of the Finnish wolf population

J. ASPI,* E. ROININEN,* M. RUOKONEN,* I. KOJOLA† and C. VILÀ‡
*Department of Biology, University of Oulu, P. O. Box 3000, FIN-90014, Oulu, Finland, †Finnish Game and Fisheries Research Institute, Oulu Game and Fisheries Research, Tutkijantie 2 E, FIN-90570 Oulu, Finland, ‡Department of Evolutionary Biology, Uppsala University, Norbyvägen 18D, S-752 36 Uppsala, Sweden

Abstract

The Finnish wolf population (*Canis lupus*) was sampled during three different periods (1996–1998, 1999–2001 and 2002–2004), and 118 individuals were genotyped with 10 microsatellite markers. Large genetic variation was found in the population despite a recent demographic bottleneck. No spatial population subdivision was found even though a significant negative relationship between genetic relatedness and geographic distance suggested isolation by distance. Very few individuals did not belong to the local wolf population as determined by assignment analyses, suggesting a low level of immigration in the population. We used the temporal approach and several statistical methods to estimate the variance effective size of the population. All methods gave similar estimates of effective population size, approximately 40 wolves. These estimates were slightly larger than the estimated census size of breeding individuals. A Bayesian model based on Markov chain Monte Carlo simulations indicated strong evidence for a long-term population decline. These results suggest that the contemporary wolf population size is roughly 8% of its historical size, and that the population decline dates back to late 19th century or early 20th century. Despite an increase of over 50% in the census size of the population during the whole study period, there was only weak evidence that the effective population size during the last period was higher than during the first. This may be caused by increased inbreeding, diminished dispersal within the population, and decreased immigration to the population during the last study period.

Keywords: bottleneck, *Canis lupus*, dispersal, isolation by distance, population decline

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Introduction

Our knowledge of the past demographic history of rare and endangered animal species is often incomplete. In providential cases historical hunting or other statistics may provide some information on the past demographic history of a population. However, even in these cases the statistics are often deficient and may only reflect the number of killed animals, which is not always correlated with population size. Fortunately, over the last decade, a number of new methods of population genetic analysis to infer demographic and history have been introduced. In particular, coalescent-based modelling has provided a powerful new means of estimating demographic parameters from patterns of multilocus variation in contemporary populations. These methods may be used to infer past demographic parameters in species with unreliably documented past history (see Beaumont 2004 for a recent review).

Based on historical documents it has been estimated that at least 23 000 wolves (*Canis lupus*) were killed in Finland during the last 150 years. Organized drives started in the middle of the 19th century (Fig. 1), and at the end of the century over 300 wolves were killed annually (Ermala 2003). The population was almost extirpated before the end of the 19th century, and by the turn of the century the wolf was present only in the eastern and northern parts of the country (Boitani 2003; Ermala 2003). Since the beginning of the 20th century the estimated average population

Correspondence: Jouni Aspi. Fax: +358-8-5531061; E-mail: jouni.aspi@oulu.fi

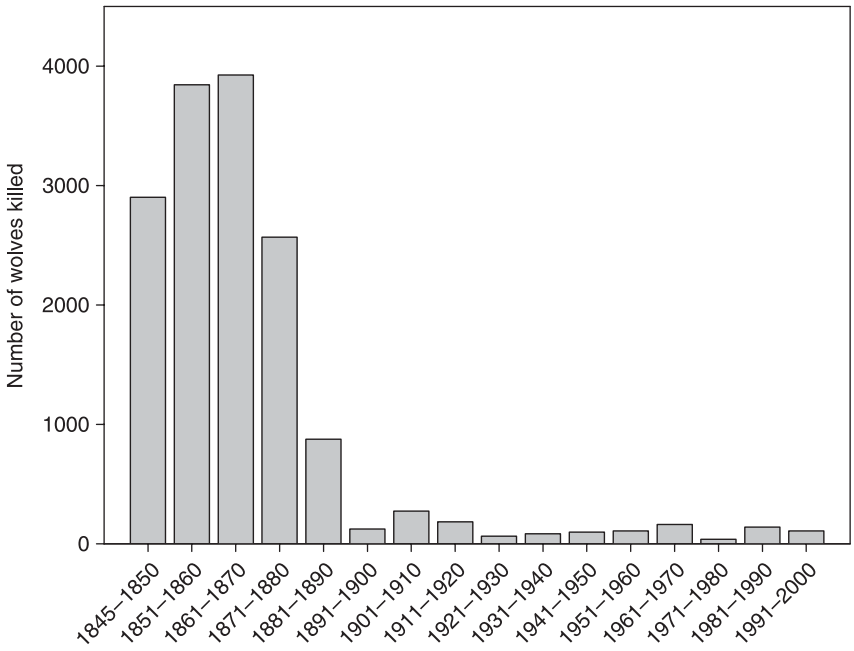

Fig. 1 Estimated number of wolves killed in Finland between 1845 and 2000 (redrawn after Ermala 2003).

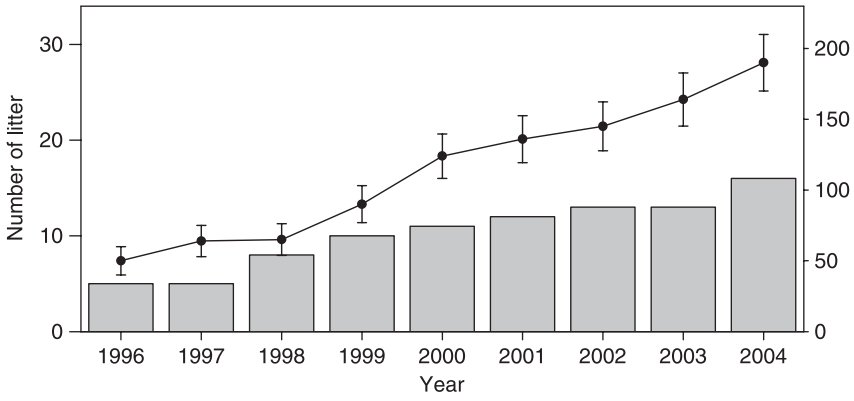

Fig. 2 Estimated wolf population size (± 95% confidence limits) (line and dot; right *y*-axis) and number of litters (bars; left *y*-axis) in Finland between 1996 and 2004 (redrawn from I. Kojola *et al.*, unpublished).

size has probably been only several tens, and fluctuations in wolf numbers in Finland have mirrored fluctuations in neighbouring Russian Karelia until the late 1990s (Pulliainen 1965, 1980; Boitani 2003). It is believed that during the 1920s and 1970s there were severe bottlenecks during which the population consisted only of a few individuals (Pulliainen 1965, 1980; Ermala 2003). Conversely, during the last decade the wolf population has increased (Fig. 2) and expanded its distribution range as a result of conservation strategies and hunting control (Kojola & Määttä 2004; Kojola *et al*. 2006). The minimum size estimate for the population has increased over 50% during the last five years (Kojola & Määttä 2004), and currently (2004) there are about 190 wolves (95% confidence range 180–200) including 17 breeding pairs in Finland. Even though the past history of the Finnish wolf is known to some extent, genetic methods could be used to complement our general view of the demographic history of the population.

A decreasing trend in census population size is almost invariably accompanied by a decrease in the effective population size (N_e). Several kinds of effective sizes have been defined (Crow & Denniston 1988). One, which is interesting in genetic conservation of species, is the variance effective number ($Ne_{(v)}$) which is defined as the size of an ideal population experiencing the same rate of genetic change as the natural population of interest (Crow & Kimura 1970; Crow & Denniston 1988). N_e is important because it determines rates of loss of genetic variation, fixation of deleterious alleles and inbreeding (Wright 1969). Therefore, early detection of N_e reduction is critical, because immediate management action may be necessary to avoid population endangerment or extinction (Schwartz *et al*. 1998). Owing to variation in family size and overlapping generations in a wolf population, N_e is probably much smaller than the census population size, N_c (cf. Frankham 1995; Nunney 1995). Even though the census size of the Finnish wolf

population is at present rather well known, the effective population size is still difficult to estimate from demographic field surveys (cf. Frankham 1995; Nunney 1995). Genetic methods may provide more effective ways for estimating N_e (for reviews see Schwartz *et al*. 1998; Tallmon *et al*. 2004).

The Finnish wolf population is not totally isolated from other wolf populations, and gene flow from neighbouring populations may have increased the effective population size and maintained genetic diversity despite population bottlenecks. The population is presumed to be connected with the nearest wolf population, in the Russian Karelia (e.g. Pulliainen 1965, 1980; Boitani 2003). This population was also almost extirpated in the first half of the 20th century, but it began to recover in the late 1950s. By the mid 1970s wolves inhabited all parts of northwestern Russia again. However, the population started to decline again after the early 1980s, from approximately 600–700 to the present 300–350 (Danilov 1996). During some periods there has been male-biased migration between the populations (Pulliainen 1965, 1980). However, it seems that at present the numbers of wolves in Finland are no longer following the fluctuations of the larger Russian Karelia population (Kojola & Määttä 2004) which suggests that the Finnish population may be becoming isolated. The next closest wolf population inhabits southern Scandinavia, more than 600 km west of the known limits of the Finnish population. Although there appears to be some migration between the Scandinavian and Finnish wolf populations, genetic investigations suggest that they are genetically differentiated. The present gene flow between the populations is negligible, and barriers to gene flow may have existed for a very long time (Ellegren 1999; Sundqvist *et al*. 2001; Flagstad *et al*. 2003; Vilà *et al*. 2003; Seddon *et al*. 2005).

Although migration may increase the effective population size, other factors may decrease it, for example population subdivision into several reproductive units. Such substructuring has been described among North American wolf populations, even within a relatively small region (Carmichael *et al*. 2001; Geffen *et al*. 2004; Weckworth *et al*. 2005). Differentiation between wolf populations seems often, but not always, associated to the presence of topographical barriers. The genetic structure of the Finnish wolf population has not been addressed thus far. However, no barriers to wolf dispersal or regular migration routes are known in Finland (e.g. Kojola *et al*. 2006). Nevertheless, Carmichael *et al*. (2001) suggested that prey specialization may also influence patterns of gene flow between wolf populations. Wolves predominantly prey on moose in the southern part of Finland, whereas in the eastern and northern parts of the country wild forest and semidomestic reindeer, respectively, make up a significant proportion of their diet (Pulliainen 1965; Gade-Jörgensen & Stagegaard 2000; Kojola *et al*. 2006). Thus, it could be possible that this difference in prey specialization may have initiated population substructuring. Moreover, isolation by distance between individuals might exist even without population structure or fragmentation. Isolation by distance between populations has been described among some (e.g. Geffen *et al*. 2004) but not all (e.g. Weckworth *et al*. 2005) North American wolf populations.

The genetic diversity in the Finnish wolf population has been previously estimated and used as a reference for other wolf populations (Flagstad *et al*. 2003; Lucchini *et al*. 2004). However, there is no comprehensive investigation of its genetic structure. The aim of this study was to explore the genetic diversity, population structure, and past demographic history of the Finnish wolves. We were also interested in estimating the variance effective size of the population and possible recent changes in it. Moreover, we also evaluated the usefulness of the new genetic methods to explore the past demographic history of an endangered species. Especially because there are several statistical estimators of effective population size, and there is not yet comprehensive conception of usefulness of different methods when variable number of samples and loci are used in populations with different effective and survey sizes, we used several different estimators to investigate their usefulness and consistency.

Materials and methods

DNA extraction and microsatellite analysis

A total of 116 tissue samples and two blood samples on snow were collected, representing a time span of 9 years. Exact geographical coordinates were available for 117 (Fig. 3). The samples were divided into three temporal groups 1996–1998, 1999–2001 and 2002–2004, each group comprising of 31, 39 and 48 individual samples, respectively. The three years difference between the midpoints of the temporal samples corresponds to the average age at which a female gives birth to her offspring in our study population (3.4 years, I. Kojola *et al*., unpublished), and has also been used as average generation time in other genetic studies (Vilà *et al*. 1999; Lucchini *et al*. 2004; Leonard *et al*. 2005). Differences between mean sampling dates were 2 years 10 months, and 3 years 2 months between the first and second, and the second and third sample, respectively. Because of population expansion, the average geographic location of the samples shifted slightly to west (the shift in the median location along east–west axis was 41 km between the first and last sample) and north (the shift in the median location was 77 km along south–north axis) during the study. However, there was no significant difference in spatial variance between the temporal samples along the north–south or east–west axis (Levene

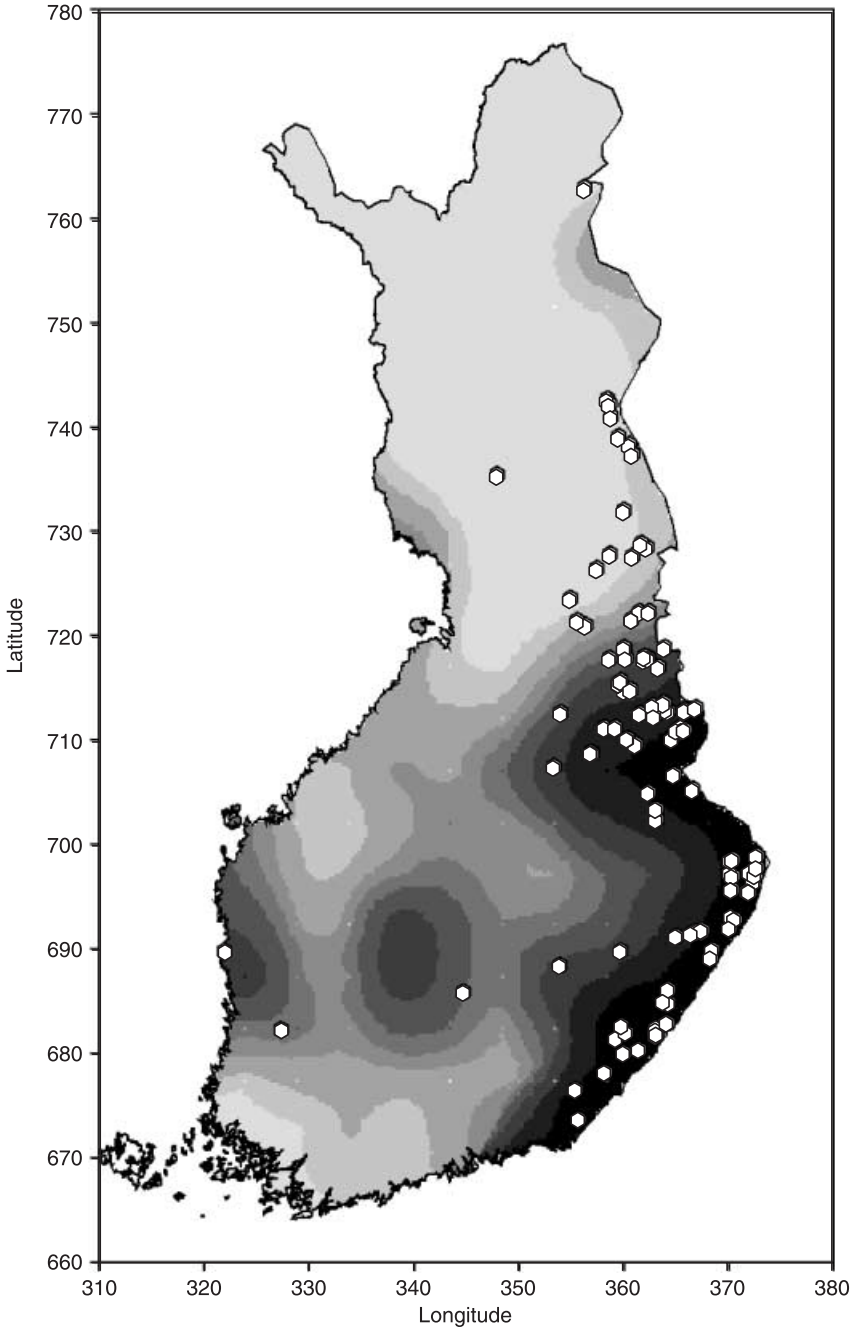

Fig. 3 Relative density of wolf population in Finland based on observations of snow tracks (different shades) in 2004 (I. Kojola, unpublished) and the geographic location of samples (white dots).

test of homogeneity of variances: $P > 0.10$ for both directions).

Genomic DNA from tissue or blood was extracted employing standard phenol–chloroform extraction protocols (32 samples) or the DNeasy® Tissue Kit (QIAGEN) (86 samples). We initially genotyped the tissue and blood samples for allelic variation at 11 autosomal microsatellite loci (Ostrander *et al*. 1993; Fredholm & Winterø 1995; Francisco *et al*. 1996) including eight dinucleotide (C20.253, CXX.109, C09.173, CXX.225, CPH2, CPH4, CPH8, CPH12) and three tetranucleotide repeats (C2001, C2088, C2096). To minimize scoring errors some samples were amplified up to three times. In the few samples where an ambiguous result still occurred, we recorded a half-locus (Miller *et al*. 2002). Negative extraction and polymerase chain reaction (PCR) controls were used throughout the study to monitor contamination.

Amplification of DNA extracts was performed using a Peltier Thermal Cycler-200 (MJ Research) in 10-μL reactions containing 20 ng of template DNA, 1 × PCR buffer (10 mM Tris-HCl 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$,

0.2 mM dNTP, 3.2 pmol of each primer, 0.5 U of DNA polymerase (Ampli*Taq* GOLD®), and sterile water. For C2088 the amount of template DNA used was 35 ng. The PCR profile was identical across all markers and included an initial denaturation step of 95 °C for 10 min, 11 touchdown cycles with 94 °C for 30 s, 58 °C for 30 s decreasing by 0.5 °C in each cycle and 72 °C for 1 min, 28 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 10 min. All PCR microsatellite products were run on an ABI 377 instrument (PerkinElmer Applied Biosystems) and gel analysis was performed using the software packages GENESCAN 3.1 and GENOTYPER 2.0 (PerkinElmer Applied Biosystems).

The program MICROCHECKER version 2.2.3 (van Oosterhout *et al*. 2004) was used to identify possible null alleles, large allele dropout, scoring errors due to stutter peaks, and possible typographic errors. The analysis indicated that null alleles may be present at the locus CXX.109 as was suggested by the general excess of homozygotes for most allele size classes in each temporal sample. The binomial test could not be conducted for the first time period because more than 50% of the alleles at this locus are of one allele size class. However, the combined probability of observed homozygote class frequencies was significantly larger than expected at level $P > 0.05$ in the two last time periods. Estimates of the frequencies of the null allele (Brookfield 1996) were constant being 0.102, 0.101 and 0.104 in the three temporal samples, respectively. Thus, we discarded this locus from all analysis. No signs of null alleles were seen at other loci.

Genetic diversity and inbreeding

We used the software GENETIX (Belkhir *et al*. 2004) to estimate observed and expected heterozygosities, number of alleles and inbreeding coefficients for each locus and temporal sample. The program provides the distribution of the parameter values by the appropriate resampling scheme of the relevant objects. We tested for linkage disequilibrium between all pairs of loci and over all loci in each temporal sample according to the method of Black & Kraftsur (1985) implemented in GENETIX. Deviations from Hardy–Weinberg equilibrium in each temporal sample were tested for by using the program GENEPOP (Raymond & Rousset 1995b). For each population–locus combination, departure from Hardy–Weinberg expectations was assessed by exact tests with unbiased P values estimated through a Markov chain method (with 1000 as dememorization number, 500 batches, and 1000 iterations per batch) and a global test across loci and populations was performed using Fisher's method (Rousset & Raymond 1995). Exact tests of population differentiation among the temporal samples were conducted as described by Raymond & Rousset (1995a) using GENEPOP.

Population structure and isolation by distance

We applied two Bayesian approaches to our pooled microsatellite data set to infer possible hidden spatial population structure in the Finnish wolf population across all time periods. First, we used the program STRUCTURE version 2 (Pritchard *et al*. 2000; see also Falush *et al*. 2003) which uses a Markov chain Monte Carlo (MCMC) approach to infer the number of populations (K) in a data set without prior information of the sampling locations. We assumed a model with population admixture and that the allele frequencies were correlated within populations (Falush *et al*. 2003). We conducted a series of independent runs (4–8 with a mean of 6.64) for each value of K (the number of populations) between 1 and 14 with a burn-in period of 50 000 iterations and collected data for 500 000 iterations. Second, we used the program BAPS version 3.1 (Corander *et al*. 2003, 2004) which jointly estimates the posterior probabilities for the number of populations, the partition of individuals among the inferred populations, and the relative allele frequencies. Contrary to STRUCTURE, BAPS 3.1 uses stochastic optimization to infer the posterior mode of the genetic structure.

We conducted an assignment analysis to get further information on the distinctiveness of the population and to identify possible first-generation migrants. Individual-based assignment tests, which assign individuals probabilistically to candidate populations by their multilocus genotype, may be used to identify individuals which do not seem to belong to a given population, and are thus possible migrants (e.g. Berry *et al*. 2004; Manel *et al*. 2005). We performed self-classification runs for the pooled temporal samples using the Rannala & Mountain (1997) Bayesian individual assignment method with the 'Leave one out' option as implemented in the program GENECLASS 2 (Piry *et al*. 2004) to estimate the likelihood that a wolf originated from the population. The marginal probability of given individual multilocus genotype was compared to the distribution of marginal probabilities of randomly generated multilocus genotypes (10 000 replicates), and if the value was below $P < 0.01$, the individual was 'rejected' from the wolf population.

Isolation by distance and resulting spatial genetic structure within a population has often been quantified as 'neighbourhood size' (Nb). Neighbourhood size is a concept originally formulated by Wright (1969) and was intended to approximate 'the population of a region of continuum from which the parents of individuals born near the centre may be treated as if drawn at random' (Wright 1969; p. 291). Neighbourhood size is usually defined as $Nb = 4\pi\sigma^2 D$, where σ^2 is the axial dispersal variance and D is the density of the population (Wright 1969). Neighbourhood size may be estimated indirectly from the slope of the regression between genetic relatedness and geographic

distance (Rousset 2000; Hardy 2003). We estimated isolation by distance for each temporal sample and for pooled data using the kinship coefficient between individuals vs. distance on a logarithmic scale (Hardy 2003) using program SPAGEDI (Hardy & Vekemans 2002, 2003). We used the Loiselle *et al*. (1995) estimator of kinship coefficient, which is especially suitable in cases when there are low frequency alleles present (Hardy & Vekemans 2003). Because there is no consensus regarding the way to generate distance classes, we used the equal frequency method, i.e. uneven lags that comprise a constant number of samples (Escudero *et al*. 2003). A jackknife procedure over loci was used to estimate standard errors for each distance class and 10 000 randomizations of individual spatial locations were performed to test for the overall spatial structure (Hardy & Vekemans 2002, 2003). To characterize the spatial genetic pattern of subpopulations we calculated the indirect estimate of neighbourhood size (*Nb*) on the basis of spatial autocorrelation. The neighbourhood size was estimated as $-(1 - F_1)/b$, where b is the slope of the regression, and F_1 is the average F_{ij} (kinship) estimate for adjacent individuals i and j (Hardy 2003; Vekemans & Hardy 2004).

Current effective population size

Currently, many different genetic methods are available to infer variance effective size of a population. Most commonly used is the so-called temporal method in which N_e is estimated from changes in gene frequencies, or the rate of coalescence of alleles between samples taken at different times (e.g. Waples 1989; Berthier *et al*. 2002). Several statistical estimators of N_e for the temporal method are available including the moment-based (Waples 1989), maximum-likelihood-based (Williamson & Slatkin 1999; Anderson *et al*. 2000), pseudo-likelihood (Wang 2001; Wang & Whitlock 2003), and Bayesian coalescent-based (Berthier *et al*. 2002; Beaumont 2003) estimators. We used all these estimators for our microsatellite data to investigate their usefulness and consistency.

We assumed that our three temporal samples represented three sequential wolf generations. NEESTIMATOR (Peel *et al*. 2004) was used to estimate the moment-based estimator of N_e. We used TMVP (Beaumont 2003), an updated version of the TM3 program developed by Berthier *et al*. (2002), to obtain a posterior distribution of N_e using an MCMC approach with importance sampling (Beaumont 2003). We assumed a model with constant population size and used 20 000 MCMC updates (an initial 10% were discarded as burn-in) with 10 updates between output estimates, and the N_e ceiling was set at 1000. MCLEEPS provides the maximum-likelihood estimator of N_e using Monte Carlo simulations (Anderson *et al*. 2000). We used this program to compute the likelihoods for N_e values between 1 and 1000, in steps of 1, and used 1000 Monte Carlo replicates for each value of N_e. Finally, MNE provided a pseudo-likelihood N_e as described in Wang (2001). We used an updated version of the program, described in Wang & Whitlock (2003), with the N_e ceiling of 1000.

Past demographic history

We used a Bayesian coalescent-based approach developed by Beaumont (1999) to assess long-term changes in historical population size. The method has previously been used by Lucchini *et al*. (2004) to estimate past demographic histories in several European wolf populations. The method provides distributions of the exponential population growth rate r, defined as the ratio of the current population size to that just prior to the period of population size change, and tf, which is the time since the population size began to change, expressed in units of the current population size. Distributions were obtained using the computer program MSVAR (Beaumont 1999), which conducts MCMC simulations. We performed the analyses for a linear model of population change because exponential model is primarily valid for short-term strong declines (Beaumont 1999). We used rectangular priors for the parameters, with bounds of (–5, +5) for $\log_{10}(u)$, $\log_{10}(r)$, and $\log_{10}(tf)$. These limits were chosen to be sufficiently broad so that the high-density region of the posterior distribution would be relatively unaffected by the prior (Storz & Beaumont 2002). We used 20 000 thinned updates and a thinning interval of 10 000 steps, leading to a total number of 2×10^8 updates (an initial 10% were discarded as burn-in). Convergence was assessed in two ways: by looking at plots of parameter values against time, and by comparing posterior distributions for parameters from seven independent runs with different starting points for the chains. For the latter method we tested that the quantity $\sqrt{(Vw + Vb)/Vw}$ (where Vw is the variance of the parameter within a chain and Vb is the variance of the means among chains) for all parameters was < 1.1 (i.e. where Vb is ~5% of Vw; see Beaumont 1999). Approximate plot densities were calculated from the sampled parameters, and the 0.9 highest posterior density (HPD) limit for each parameter was estimated.

A short-term change in effective population size was analysed using the TMVP procedure (Beaumont 2003). When applied to the problem of estimating recent changes in effective population size from temporally spaced gene frequency data, the method gives the posterior distribution of effective population size at the time of the oldest sample (N_A) and at the time of the most recent sample (N_0), assuming a model of exponential growth or decline during the interval. The program samples independent genealogical histories using importance sampling and then samples other parameters with Markov chain Monte Carlo simulations. We ran 20 000 MCMC updates (an initial 10% were

discarded as burn-in) with 10 updates between estimate outputs, and used a rectangular prior of 0–1000 for both N_A and N_0. In addition we used Hill's (1981) one sample method to estimate the effective population size for each temporal sample to infer short-term changes in N_e. This method uses associations among alleles at different loci to infer N_e, and assumes that linkage disequilibrium is produced by drift in a small population among unlinked loci. However, linkage equilibrium-based estimates should be interpreted cautiously, because Bartley *et al*. (1992) have shown that sample sizes over 90 may be necessary to obtain precise estimates of N_e when using this method. We used the program NEESTIMATOR Peel *et al*. 2004) to estimate this linkage equilibrium-based estimator of N_e for each temporal sample.

Population bottlenecks can produce distinctive genetic signatures in the distributions of allele size and expected heterozygosity (Cornuet & Luikart 1996; Luikart & Cornuet 1998; Garza & Williamson 2001). When a population experiences a reduction of its effective size, it generally develops excess gene diversity at selectively neutral loci, i.e. the gene diversity computed from a sample of genes is larger than the gene diversity expected from the number of alleles found in the sample of a constant-size population. This condition occurs because the rare alleles that were lost contributed little to the overall heterozygosity (Cornuet & Luikart 1996). Population bottlenecks may also initiate gaps in the size distribution of microsatellite alleles (Garza & Williamson 2001). We assessed the wolf population for a deficiency of low frequency allele classes by examining the overall distribution of allele frequency classes ('mode shift' test) and using Wilcoxon test as implemented in the program BOTTLENECK (Cornuet & Luikart 1996) under the two-phase mutation model with 95% single-step mutations. The gaps in distributions can be quantified as the *M* ratio, the mean ratio of the number of alleles to the allele size range across all loci (Garza & Williamson 2001). Means of *M* ratios were calculated for each temporal sample using AGARST (Harley 2004).

Results

Genetic diversity and inbreeding

The overall genetic differences (Raymond & Rousset 1995a) between the temporal samples were highly significant ($\chi^2 = 82.14$, d.f. = 20, $P < 0.0001$), as they were for each pair of samples ($P \leq 0.0007$ in all cases).

The average number of alleles (Table 1) was very similar in each temporal sample varying from 5.3 to 5.6 (note that allele numbers are not corrected for differences in sample size). The observed heterozygosity in the first (1996–1998) and second (1999–2001) temporal samples were identical (0.706 ± 0.105 and 0.706 ± 0.091, respectively) whereas in the last sample the observed heterozygosity was slightly, although not significantly, lower (0.680 ± 0.088). The expected heterozygosity was lower than the observed heterozygosity in the first two temporal samples (0.664 ± 0.076 and 0.663 ± 0.072, respectively), suggesting an excess of heterozygotes. Inbreeding coefficients in both the first ($F = -0.045$; 95% confidence limits: –0.156 to 0.016) and the second ($F = -0.052$; 95% confidence limits: –0.102 to –0.031) temporal samples were negative. However, only in the latter sample were both 95% bootstrapped (1000 permutations) confidence limits negative, indicating significant inbreeding avoidance within the wolf population. In the most recent sample (2002–2004) the expected heterozygosity (0.691 ± 0.066) was higher than the observed one, and the inbreeding coefficient was positive ($F = 0.029$), although not significantly (95% confidence limits: –0.052 to

Table 1 Expected (H_E) and observed (H_O) heterozygosities, number of alleles (*A*) and inbreeding coefficient (*F*) in the studied microsatellite loci in the three temporal samples of the wolf population

	1996–1998 (*N* = 31)				1999–2001 (*N* = 39)				2002–2004 (*N* = 48)			
Locus	H_E	H_O	*A*	*F*	H_E	H_O	*A*	*F*	H_E	H_O	*A*	*F*
C20.253	0.760	0.867	6	–0.124	0.772	0.816	7	–0.144	0.797	0.703	6	0.125
C2001	0.650	0.774	4	–0.174	0.677	0.821	6	–0.199*	0.727	0.721	6	0.008
C2088	0.673	0.667	5	0.027	0.706	0.790	7	–0.105	0.613	0.683	5	–0.119
C2096	0.665	0.722	4	–0.057	0.628	0.750	5	–0.181*	0.698	0.625	7	0.131
C09.173	0.685	0.807	8	–0.162	0.548	0.595	3	–0.071	0.647	0.786	5	–0.201*
CXX.225	0.672	0.774	4	–0.137	0.642	0.611	3	0.063	0.645	0.634	3	0.054
CPH2	0.721	0.645	5	0.122	0.693	0.579	5	0.177	0.697	0.591	6	0.152
CPH4	0.693	0.679	5	0.039	0.743	0.692	5	0.081	0.747	0.643	5	0.076
CPH8	0.652	0.630	8	0.054	0.562	0.743	7	–0.308*	0.760	0.833	7	–0.095
CPH12	0.471	0.500	6	–0.044	0.658	0.667	5	0.013	0.580	0.553	5	0.059
Mean	0.664	0.706	5.6	–0.045	0.663	0.706	5.3	–0.052*	0.691	0.680	5.4	0.029

*$P < 0.05$.

0.080). We did not find any significant overall deviation from Hardy–Weinberg equilibrium proportions in the two first samples ($\chi^2 = 18.04$, d.f. = 20, $P = 0.585$ and $\chi^2 = 17.66$, d.f. = 20, $P = 0.609$, respectively). However, the most recent sample significantly deviated from equilibrium ($\chi^2 = 36.05$, d.f. = 20, $P = 0.015$). There was a significant ($P < 0.05$) deficit of heterozygotes in 2 of the 10 loci, suggesting that inbreeding in the population may have increased despite that the inbreeding coefficient was not significantly different from zero. When all temporal samples were pooled, no significant deviation from Hardy–Weinberg equilibrium proportions was found ($\chi^2 = 71.75$, d.f. = 58, $P = 0.11$).

No significant overall linkage disequilibrium was found in the first ($\chi^2 = 10.00$, d.f. = 15, $P = 0.82$) and second temporal samples ($\chi^2 = 23.44$, d.f. = 30, $P = 0.80$), or in the pooled data set ($\chi^2 = 4.87$, d.f. = 42, $P = 0.22$). However, in the most recent sample, we found significant linkage disequilibrium between loci ($\chi^2 = 45.84$, d.f. = 25, $P = 0.01$).

Population structure and isolation by distance

Both Bayesian approaches suggested that the wolf microsatellite data show the existence of more than one cluster. The only STRUCTURE model that explained the data sufficiently ($P = 0.9$) was the model with $K = 5$, although the model with $K = 9$ also had a low probability ($P = 0.1$). The other STRUCTURE models did not explain the data well ($P < 0.001$ in each case). On the other hand, the most probable numbers of clusters obtained when using the program BAPS were $K = 11$ ($P = 0.945$) and $K = 12$ ($P = 0.055$). Even though the number of clusters varied between approaches, it appeared that when the wolf individuals were assigned to the most probable number of clusters using either of the programs, there was no clear spatial pattern among the clusters. The clusters tended to overlap broadly and some of the clusters had a very wide geographical distribution (data not shown). In both cases the suggested clusters consisted mainly of the members of known family groups. Accordingly, it seems that even though the wolf population did not form a single reproductive unit, there is not clear spatial subdivision within the population and the suggested clusters seem to represent different 'family lines'.

The self-classification assignment tests showed that only 4 wolf individuals out of 118 (3%) were not assigned correctly to the Finnish population. Two of these individuals were sampled during the first temporal period (one from 1997 and other 1998), and one from each of the later samples (1999 and 2003). All of them were killed or found dead around the Finnish-Russian border. One of the individuals was a female and the rest were males. Although sex-biased dispersal in wolves is not well documented (Mech & Boitani 2003), the observed pattern is consistent with the male-biased dispersal described by Pulliainen (1965, 1980) and inferred by Flagstad *et al*. (2003) using genetic methods.

While the Bayesian approaches did not find any spatial substructuring within the Finnish wolf population, the spatial autocorrelation analysis suggested local genetic structure within the population. The negative regression slope ($b = -0.021$) between kinship coefficient and logarithmic distance between individuals was significant ($P < 0.001$). There was significant deviation from the population mean kinship estimate in the closest and most distant distance classes (except in the last one) (Fig. 4). Positive values of kinship coefficient were found at short distances, meaning that neighbouring individuals had a

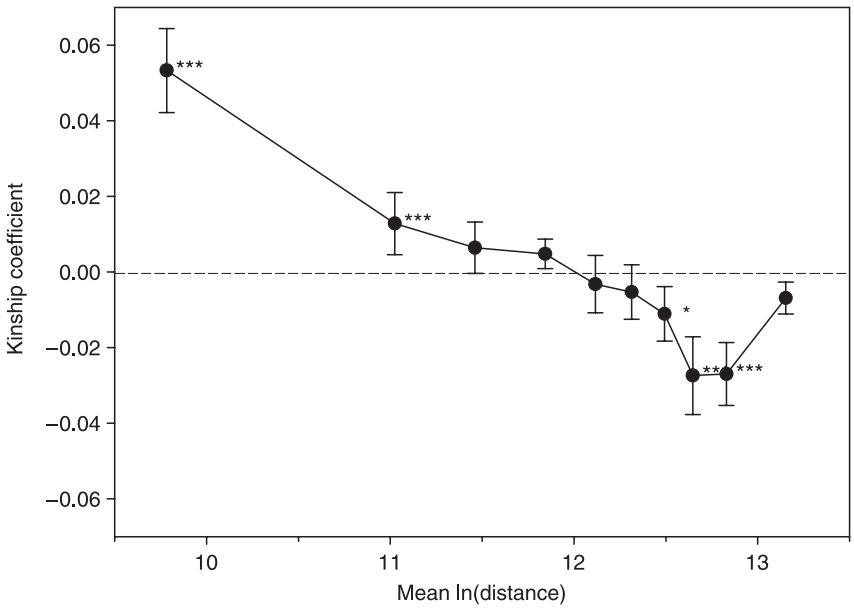

Fig. 4 Kinship coefficient vs. logarithmic distance between individuals in the wolf population. The asterisks represent significant deviation of a distance class from the population mean: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

higher genetic relatedness than random pairs of individuals, whereas negative values of kinship occurred at larger distances, indicating isolation by distance within a population. However, in the last distance class the mean kinship estimate was not significantly lower than the population mean estimate, and was even slightly lower than in the preceding distance classes. This class represents wolves which have dispersed furthest, to the formerly uninhabited areas and in the nonrelevant range of distances with respect to the isolation-by-distance concept (cf. Vekemans & Hardy 2004). The intercept of the correlogram with the *x*-axis was approximately 163 km (Fig. 4) suggesting that within this distance the wolf individuals are more related than on average in the population. It has been suggested that this 'patch width' could be used as a guideline to define meaningful conservation units in a continuous population (e.g. Diniz-Filho & Telles 2002). However, as shown recently by Fenster *et al*. (2003), this 'patch width' is not necessarily characteristic of the populations studied, as it seems to depend strongly on a sampling scheme.

The neigbourhood size (Nb) estimated from the slope and the average kinship between adjacent individuals was 44.5, and given that the density of the population is about 3 wolves per 1000 km^2, the neigbourhood area (Na) was about 14 900 km^2. Assuming that the axial dispersal distances are normally distributed, and that the population density (D) is about 3 individuals/1000 km^2, we estimated (using the equation $Nb = 4\pi\sigma^2 D$) that the average dispersal distance for wolves in this population is 97.2 km. When estimated separately for each temporal sample the regression slope between kinship coefficient and logarithmic distance between individuals was negative and significant ($P < 0.001$, 10 000 permutations). The slope and, correspondingly, the neighbourhood size, were very similar in the first ($b = -0.011$; $Nb = 55.6$) and second ($b = -0.013$; $Nb = 56.2$) samples. However, in the last sample the slope of the regression was steeper and thus the neighbourhood size was smaller ($b = -0.030$; $Nb = 30.2$) suggesting that dispersal distances decreased during the last period.

Current effective population size

All the programs gave very similar estimates for the effective population size of about 40 wolves, ranging from 37.8 to 43 (Table 2). Each estimate was slightly larger than the present estimate of the number of breeding individuals ($N_B = 34$). The harmonic mean of the number of breeding individuals (2 × number of known litters; Fig. 2) during the study period was 15.2 individuals. This value is outside the confidence limits of all estimates. Given that the harmonic mean of the census of the Finnish wolf population during the study period has been 94, this would suggest a ratio of 0.42 (40/94) between effective and census population sizes (N_e/N_c).

Past demographic history

The results of Beaumont (1999) procedure for assessing population decline or expansion strongly supported a long-term decline in the wolf population (Fig. 5). All sampled points of $\log_{10}(r)$ were substantially below zero in all seven replicates, with an average mode of –1.14 and 90% HPD interval of from –1.572 to –0.781 suggesting strongly that the ancient population size was larger than the contemporary size. From $r = 0.08$ ($= N_0/N_A$), computed as the antilog of $\log_{10}(r)$, we could estimate that the contemporary wolf population size is roughly 8% (range 3–19%) of its historical size. Given that the current effective size is about 40 wolves, we may thus estimate that the ancient effective size was about 590 wolves. Assuming that the ratio of the N_e/N_C in the ancient population was similar to that in the contemporary population (40/94), there could have been almost 1400 wolves in Finland a few hundred years ago. The average mode of $\log_{10}(tf)$ was –0.414 with a 90% HPD interval of –0.676 to 0.164, suggesting that the wolf population started to decline 0.39 N_0 generations ago (range: 0.21–0.69 N_0). Assuming that the current population size is $N_e = 40$ wolves (corresponding to $N_0 = 80$, measured as number of chromosomes) and generation time is 4 years we might estimate that the population decline dates to the late 19th century (1875; range 1780–1932) whereas a generation time of 3 years would suggest an early 20th century begin of decline (1913; range 1835–1949).

The simulated posterior distributions of the 'ancestral' (1996–1998) and 'current' (2002–2004) wolf population size suggest that there is very little evidence for a short-term change in population size (Fig. 6). The modes and

Table 2 Effective population size estimates and their approximate confidence limits of the wolf population based on the different temporal methods

Method	N_e	Confidence limits	Program	References
Moment based	39.5	19.3–98.7	NeEstimator	Waples (1989), Peel *et al*. (2004)
Coalescence MCMC	40.0	31.0–58.0	tmvp	Berthier *et al*. (2002), Beaumont (2003)
MC likelihood	43.0	31.0–74.0	mcleeps	Anderson *et al*. (2000)
Pseudo-likelihood	37.8	25.4–63.7	Mne	Wang (2001), Wang & Whitlock (2003)

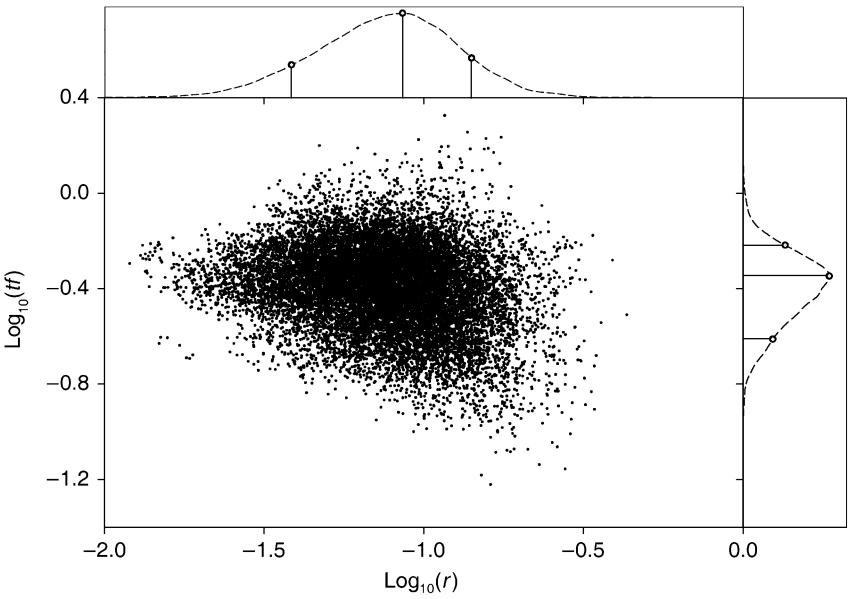

Fig. 5 Plot of the 20 000 simulated points from the marginal posterior distributions of $\log_{10}(r)$ and $\log_{10}(tf)$ for the wolf microsatellite data. In the panels on the top and right, the dotted lines give the density plots of the above parameters, and the solid lines give the 0.9, 0.5, and 0.1 HPD limits.

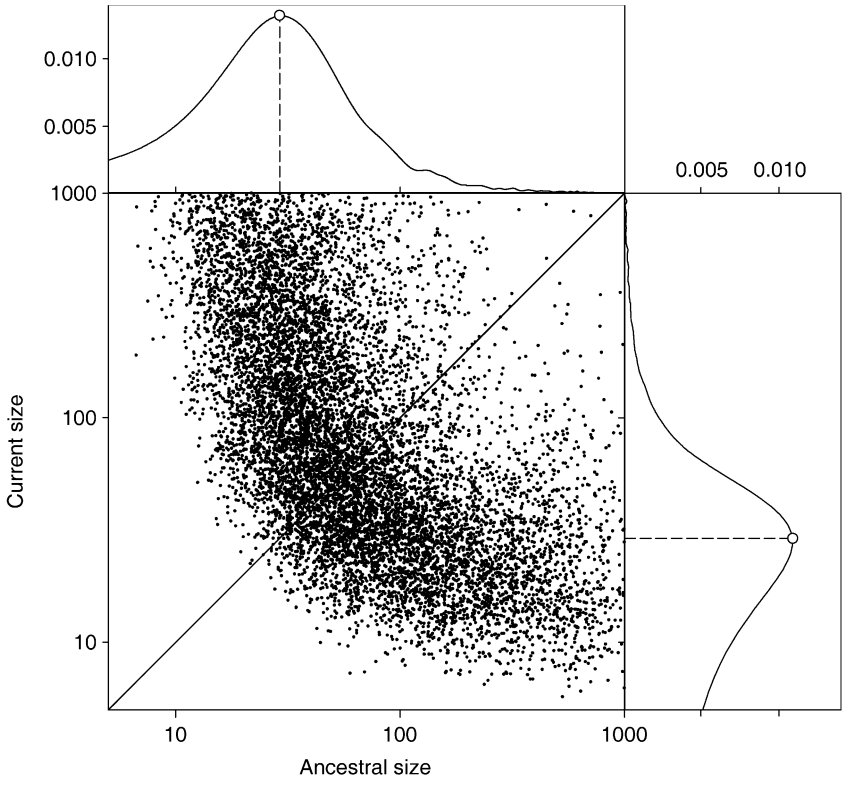

Fig. 6 Plot of the 20 000 simulated points from the posterior distributions of the 'ancestral' (N_A, 1996–1998) and current (N_0, 2002–2004) population size for the wolf microsatellite data. The line where $N_A = N_0$ is shown. In the panels on the top and right, the solid lines give the density plots of the above parameters, and the dotted lines give the 0.5 HPD limits.

90% HPD limits were 29 (19–303) and 29 (15–272) for N_A and N_0, respectively. The Bayesian factor favouring a model of population growth vs. decline (i.e. proportion of MCMC iterations where $N_0 > N_A$ divided by the proportion of iterations where $N_0 < N_A$) was 1.07 indicating also that there was only very weak evidence of population growth. The estimates of effective population size for each temporal sample based on linkage disequilibrium (Hill 1981) also did not provide support for population growth. The estimated effective population sizes (and the approximate confidence limits) for the three periods were 25.1 (19.7–33.4), 14.9 (2.8–17.5) and 10.8 (9.5–12.4), respectively, suggesting that the effective population size may have even decreased.

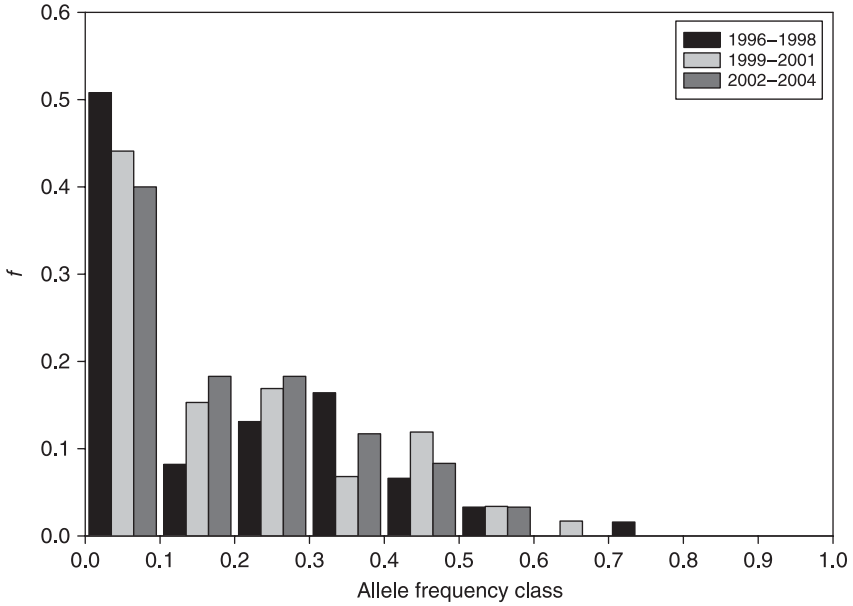

Fig. 7 Allele frequency distributions of the microsatellite loci in three temporal wolf samples.

We did not find very much evidence of past population bottlenecks in the allele frequency distributions. The allele frequencies of all three temporal samples had a normal L-shaped distribution (Fig. 7), and we did not detect significant heterozygote excess in the first two samples. However, there seems to be a decrease in the frequency of rare alleles. The frequency of the rarest allele class (< 0.1) decreased steadily from 0.51 to 0.4 during the study period (Fig. 7), and heterozygosity was higher than expected in the last temporal sample (Wilcoxon test; $P = 0.050$). The *M* ratio test to investigate gaps in the allele frequency distribution provided inconclusive results. Garza & Williamson (2001) suggested that values of *M* lower than 0.7 would indicate evidence of a bottleneck, whereas values greater than 0.8 would denote no bottleneck history. In our data set the *M* values in the first, second and third sample were between these limits, being 0.71 (± 0.21), 0.73 (± 0.22) and 0.72 (± 0.29), respectively. However, as shown by Guinand & Scribner (2003), single values of the *M* ratio are not always sufficient to unambiguously infer a bottleneck without knowledge of mutation rates and effective population sizes.

Discussion

Despite the historically documented bottlenecks in the Finnish wolf population, we found high amounts of genetic variation. Observed heterozygosities in the temporal samples varied between 0.706 and 0.680, and the expected heterozygosities were between 0.663 and 0.691. We did not find significant differences in the amount of genetic diversity between our temporal samples, even though our estimate of observed heterozygosity in the last sample was lower than among the earlier ones. Genetic diversity for the Finnish wolf population has been estimated earlier as a reference for other wolf populations. These earlier estimates seem to be very similar to ours despite the use of different microsatellite markers. Flagstad *et al*. (2003) estimated that the observed and expected heterozygosities for 'contemporary' Finnish wolves ($N = 22$) were 0.69 and 0.72, and according the Lucchini *et al*. (2004) the observed and expected heterozygosities for their sample ($N = 13$) were 0.69 and 0.73. Interestingly, in both of these studies the expected heterozygosity was lower than the observed one, as is the case for the first two of our sampling periods. Although the date of collection of their samples is not described, it is most likely that they correspond to our earlier time periods. The genetic diversity of Finnish wolves seems to be similar to other eastern European wolf populations (expected heterozygosity 0.69–0.71; Lucchini *et al*. 2004), slightly higher than most of the North American populations (expected heterozygosity 0.46–0.72; see Wayne & Vilà 2003; Weckworth *et al*. 2005), and much larger than in the isolated Scandinavian, Spanish and Italian populations (expected heterozygosity 0.49–0.60; Wayne & Vilà 2003; Lucchini *et al*. 2004).

We did not find evidence of inbreeding in the Finnish wolf population during the early phases of the study period. On the contrary, the inbreeding coefficient was negative in the first two samples. Nevertheless, the inbreeding coefficient became positive during the last period. Based on the regression line presented by Liberg *et al*. (2005), we would expect about 5% inbreeding depression in juvenile survival when $F = 0.029$. Nevertheless, the estimate of inbreeding coefficient during the last period is

still relatively low compared to the estimates of some isolated European populations (for example F is 0.10 in Italian and 0.17 in Spanish wolf populations; Lucchini *et al*. 2004) suggesting that inbreeding in the Finnish wolf population is still not severe. However, large wolf populations may be spatially structured, and in that case a large inbreeding coefficient may just be due to a Wahlund effect, i.e. reduced heterozygosity in populations due to subpopulation structure.

We did not find any spatial geographical structure in our wolf population, even though the Bayesian coalescent-based approaches suggested that there may be more than one breeding unit in the population. This was probably caused by sampling multiple individuals from the same family groups in a population that is continuous. The first two temporal samples and also the pooled data were in Hardy–Weinberg equilibrium, suggesting that the family structure did not lead to strong deviations from random mating expectations. Nevertheless, we found significant isolation by distance at the individual level on a rather restricted spatial scale. Our estimates of neighbourhood size (44.5 individuals) and area (14 900 km^2) in the pooled sample were relatively small. The very similar estimate of neighbourhood size and effective population size suggested little differentiation within the population despite the evident isolation by distance. Our estimate of mean dispersal distance was 97.3 km. Using radio-tracking data, Kojola *et al*. (2006) have estimated a very similar median dispersal distance within the Finnish wolf population of 98.5 km (range 35–445 km). However, because our estimate of the neighbourhood size was smaller during the last period as compared to the former ones, it seems that dispersal distances have become shorter and there is increasing differentiation within the population. This could have led to an increase in the inbreeding coefficient, because the breeding probability is no longer totally random within the population. The reason for these results does not seem to derive from some form of sampling bias (see sampling distribution in the Material and methods section). Although the reason for this change in behaviour is not clear, the population had at that time reached the highest population density since the end of the 19th century (Figs 1 and 2) and this may have somehow reduced the dispersal abilities of wolves. Thus far isolation by distance at an individual level has not been described in any wolf population, even though it has been described between populations at a continental level (Geffen *et al*. 2004). Our results emphasize that although wolves are capable of dispersal movements of 100s or even up to 1000 kilometres (Fritts 1983; Wabakken *et al*. 2001), the average dispersal distances, at least in sparsely populated areas, seem to be rather short. Consequently, the extirpation of wolves from part of their range is more likely to lead to losses of genetic diversity than initially suspected (Leonard *et al*. 2005).

The allele frequency distributions and observed vs. expected heterozygosities (e.g. Cornuet & Luikart 1996) did not suggest past population bottlenecks in the population. The tests for heterozygosity excess and the test based on frequency distributions can detect bottlenecks for only a narrow window of time after a bottleneck has started (Cornuet & Luikart 1996; Garza & Williamson 2001). However, Cornuet & Luikart (1996) estimated that a bottleneck of $N_e = 50$ is likely to be detectable with the heterozygote excess method for 25–250 generations ($0.25–2.5 \times 2N_e$) after the initiation of a population reduction, and M ratios should also achieve a new equilibrium only after a few hundred generations (Garza & Williamson 2001). Thus the suggested population bottlenecks in the 1920s and 1970s should still be detectable in the allele frequency distribution of the Finnish wolf population. On the other hand, both tests rely on the assumption that each sample is representative of a well-defined population with no immigration and no population substructure. If there has been migration these methods may not be able to show evidence of past bottlenecks. The observed decrease in the frequency of the rarest alleles class, together with the significant excess of heterozygosity in the last temporal sample, might suggest lowered immigration into the population, allowing the former genetic change initiated by the bottlenecks to be seen because of ceased flow of rare alleles outside the population.

The Bayesian approach of Beaumont (1999) to assess population decline strongly supported a long-term decline in the wolf population size. Our analysis suggested that there has been an almost 15-fold reduction in population size, and that the population census size may have been over 1400 wolves prior to the period of population size change. Our analyses suggested also that the population decline dates to the late 19th or early 20th century. Kojola (unpublished) has estimated that the prior decline census population size was about 800 wolves, and according to Ermala (2003), the population started to decline by the middle of the 19th century. In conclusion, the analyses of the wolf microsatellite data suggest that the population may have started to decline slightly later than thought and suffered a deeper decline than the earlier estimates imply. On the other hand, if there has been significant migration during the decline from the Russian Karelian population (which started to decline somewhat later than the Finnish population), gene flow may have delayed the change of the genetic composition, thus explaining the differences between estimates of date of decline. Immigration may also have increased the estimate of the historical population size, and in reality the numbers of the Finnish wolf population may have been somewhat lower than our analyses suggest. In the Finnish wolf population the Bayesian approach provides a general view of the demographic history, which seems to be consistent with known historical

statistics (Ermala 2003). Similar results have been obtained for other large mammalian species (Beaumont 1999; Lucchini *et al*. 2004).

Despite an increase in the number of breeding wolves in Finland almost every year (Fig. 2), the genetic approaches did not identify any recent population expansion. The posterior distributions of wolf population size at the first and last period generated by the TMVP simulations did not suggest any change in population size. The linkage disequilibrium method did not give support for the population expansion either, and even suggested a decline in population size. However, the decreasing trend may be erroneous because linkage disequilibrium may be generated by many factors, including inbreeding and an increase in the degree of differentiation inside the population. Thus, increased inbreeding, and not drift, could have caused the declining values of the estimator. Nevertheless, the contradictory census and effective size estimates suggest that either (i) the Finnish population size has actually been larger than the estimated census size during the early phase of the study period, and the larger estimated census size in the latter phase reflects only improved census methods, or (ii) even though the census size has increased, for some reason the effective size has not increased. The first explanation is not very probable, because the census methods during the entire study period have been similar. Also, a similar growth has been detected in other wolf populations in Western Europe (Wabakken *et al*. 2001; Boitani 2003).

Discrepancies between estimates of census numbers and marker-based estimate of N_e have been observed in other large-bodied terrestrial vertebrates with continental distributions (e.g. Frankham *et al*. 2002). For example, the inferred demographic history of the Finnish wolves seems to be remarkably similar to that of savannah baboons in eastern Africa (Storz *et al*. 2002a,b). In both cases the genetic estimates of N_e actually exceeded the estimated census number of the contemporary populations. In the case of the savannah baboons, the authors suggested that this may reflect a time lag between a recent reduction in census numbers and the increase in homozygosity due to drift. This explanation may be plausible also for Finnish wolves. However, other more probable factors which may have decreased the effective population size despite an increasing census size could have been increased levels of inbreeding together with decreased dispersal and immigration.

Different statistical methods provided very similar estimates of effective population size for the Finnish wolf population of approximately 40 wolves. Even though the performance of different estimators seem to vary in simulation studies (e.g. Anderson *et al*. 2000; Berthier *et al*. 2002; Tallmon *et al*. 2004), in this case study all estimators seem to perform equally well. The estimates were slightly larger than the present estimate of the number of breeding individuals (N_B = 34) and clearly larger than the harmonic mean of the number of breeding individuals during the study period (N_B = 15.2). This may suggest that some factors — like inbreeding avoidance or immigration — might have increased the effective population size of the population. On the other hand, some assumptions of the models were violated. For example, our wolf population is not an isolated entity, which vitiates one of the assumptions of the temporal method. However, our assignment analysis suggests that immigration may not have had a very large effect. Another assumption in most of the temporal methods is that the effective population size between the samples is constant. The observed increase in the census size of the wolf population suggests that this assumption may also be violated. However, the Bayesian coalescent-based analysis of temporal change and the linkage disequilibrium based N_E estimators for different time periods, did not find a change in the effective population size during the study period.

Information on effective population size for the Finnish wolves is especially important at this time because a national management plan for the wolf is currently being prepared. If the effective population size of the Finnish wolf population is about 40 wolves, as our analysis suggests, it is too small to avoid considerable inbreeding depression in the long term. Frankham *et al*. (2002) have estimated that to retain reproductive fitness, the required population size should be much greater than an effective size of 50. Accordingly, the size of the Finnish wolf population is too small to be self-sustained, even when the effect of immigration increasing N_e is taken into consideration. If the apparent immigration ceases for some reason, and the number of breeding individuals were to remain about the current 30, even in an idealized population (see, e.g. Frankham *et al*. 2002; p. 189) we would expect inbreeding to increase by 1.7% per generation. This suggests that restoring migration across the borders may be essential for the long-term survival of the population.

The Finnish wolf population has been assumed to be connected with the Russian Karelian wolf population, and immigration from the east to the Finnish population has been assumed to be quite considerable (e.g. Pulliainen 1965, 1980; Boitani 2003). In our assignment analysis only 3% of wolves seemed to be possible first generation migrants. The low number of migrants detected, together with our estimate of a relatively short dispersal distance, suggests that immigration between these populations may not be as frequent as commonly assumed. Another possibility is that the amount of migration has been larger in the past and decreased recently. This was supported by identification of most assumed migrants in the early phase of the study period. One obvious reason for possible reduced migration is the decline in the population size of the wolf in Russian Karelia (Danilov 1996). Another mechanism

behind the decreased migration rate may be more intense territorial space utilization among Finnish wolves. The stronghold of the Finnish wolf population is in the eastern part of the country (Fig. 3). Although suitable habitat with abundant prey for wolves exists further west, human pressure is higher there, which slows down the expansion of wolves in that direction. Because of recent population growth, the relative area used by occupied territories has increased, and at present the territories are effectively filling all available space at the eastern border. Since wolf packs can be highly territorial and often kill lone wolves within their territories (e.g. Packard 2003), dispersal from the east into areas occupied by other wolf packs may have been reduced. On the other hand, our assignment analysis was based only on self classification, and we did not have any samples for comparison from Russian Karelia in this study. Thus, the power of the assignment analysis may have been low, and the true number of immigrants may be somewhat larger. Accordingly, we would need to conduct a parallel study in Russian Karelia to confirm these hypotheses.

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This study is part of the PhD research of Eeva Roininen and is integrated in a larger conservation genetics project on Finnish carnivores carried out at the Department of Biology, University of Oulu, led by Jouni Aspi. Minna Ruokonen is a geneticist interested in conservation of animal species. Ilpo Kojola is a large carnivore researcher at the Finnish Game and Fisheries Research Institute. Carles Vilà is a research associate working on conservation genetics and domestication at Uppsala University.
