Conserv Genet (2011) 12:701–712
DOI 10.1007/s10592-010-0177-6

RESEARCH ARTICLE

Signatures of demographic bottlenecks in European wolf populations

Natalia Sastre · Carles Vilà · María Salinas · Vladimir V. Bologov · Vicente Urios · Armand Sánchez · Olga Francino · Oscar Ramírez

Received: 4 April 2010 / Accepted: 14 December 2010 / Published online: 24 December 2010

Abstract Monitoring the loss of genetic diversity in wild populations after a bottleneck event is a priority in conservation and management plans. Here, we used diverse molecular markers to search for signatures of demographic bottlenecks in two wolf populations; an isolated population from the Iberian Peninsula and a non-isolated population from European Russia. Autosomal, mtDNA and Y-chromosomal diversity and the effective population size (N_e) were significantly lower in the Iberian population. Neutrality tests using mtDNA sequences, such as R_2, Fu and Li's F*, Tajima's D and Fu's F_s, were positively significant in the Iberian population, suggesting a population decline, but were not significant for the Russian population, likely due to its larger effective population size. However, three tests using autosomal data confirmed the occurrence of the genetic bottleneck in both populations. The *M*-ratio test was the only one providing significant results for both populations. Given the lack of consistency among the different tests, we recommend using multiple approaches to investigate possible past bottlenecks. The small effective population size (about 50) in the Iberian Peninsula compared to the presumed extant population size could indicate that the bottleneck was more powerful than initially suspected or an overestimation of the current population. The risks associated with small effective population sizes suggest that the genetic change in this population should be closely monitored in the future. On the other hand, the relatively small effective population size for Russian wolves (a few hundred individuals) could indicate some fragmentation, contrary to what is commonly assumed.

Keywords *Canis lupus* · mtDNA · Neutrality · Y-chromosome · Autosomal microsatellites · Effective population size · European wolf

N. Sastre (✉) · M. Salinas · A. Sánchez · O. Francino · O. Ramírez
Servei Veterinari de Genètica Molecular. Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
e-mail: natalia.sastre@uab.cat

C. Vilà
Estación Biológica de Doñana-CSIC, Avd Américo Vespucio s/n, 41092 Sevilla, Spain

V. V. Bologov
Central Forest State Natural Biosphere Reserve, P/o Zapovednik, Nelidovskij rajon, 172513 Tverskaja Oblast, Russia

V. Urios
Centro Iberoamericano de la Biodiversidad (CIBIO), Universidad de Alicante, 03080 Alicante, Spain

O. Ramírez
Institut de Biologia Evolutiva, UPF-CSIC, Parc de Recerca Biomedica de Barcelona, 08003 Barcelona, Spain

Introduction

The decline of free ranging wildlife populations is frequently attributable to human persecution, degradation of habitat, disappearance of resources and/or disease, leading to endangerment or even to extinction. In a population that has gone through a demographic bottleneck, the risk of extinction is related to the reduction in genetic diversity and subsequent increase in the detrimental effects of inbreeding, decreasing the potential for adaptation (Saccheri et al. 1999; Reed et al. 2002). Therefore, the detection of bottlenecked populations is important for the implementation of suitable conservation and management plans.

Using molecular markers such as microsatellites, three simple methods are commonly applied to detect past

bottleneck events; heterozygosity excess, mode-shift and *M*-ratio tests (Cornuet and Luikart 1996; Luikart and Cornuet 1997; Garza and Williamson 2001). The heterozygosity excess approach is based on the expectation that in a bottlenecked population, as allele numbers are reduced faster than gene diversity, the observed heterozygosity will be higher than expected for a population at mutation-drift equilibrium (Cornuet and Luikart 1996). Note that this heterozygosity excess should not be confused with the excess of heterozygotes resulting from deviations from Hardy–Weinberg equilibrium expectations. The mode-shift indicator approach is based on detecting changes in allele frequency distributions considering that in a bottlenecked population, alleles with intermediate frequency will be most abundant because of rare allele loss (Luikart et al. 1998). Finally, the *M*-ratio test, relates the total number of alleles (k) and the overall range in allele sizes (r). Assuming that k is reduced more quickly than r during a bottleneck event, the ratio $M = k/r$ will be smaller in populations that have suffered a decline than in populations that are in mutation-drift equilibrium (Garza and Williamson 2001). The *M*-ratio test can distinguish between bottlenecked populations and those which have been small for a long time (Garza and Williamson 2001). Furthermore, *M*-ratio test detects changes in N_e up to 100 generations ago whereas the heterozygosity excess and mode-shift tests detect recent bottlenecks.

However, demographic bottlenecks do not always leave a genetic signature that can be detected using autosomally inherited markers because migration and/or mutation may erase bottleneck signatures in just a few generations (Keller et al. 2001; Busch et al. 2007). Moreover, in a bottleneck affecting each sex in a different way, an unequal reduction might be expected in effective population size N_e depending on the marker used (Fay and Wu 1999). For this reason, maternally (mitochondrial) and/or paternally (Y-chromosome) inherited genetic markers should be analyzed simultaneously to gain a better understanding of the population history and/or diversity. Here, we have used mitochondrial DNA (mtDNA) sequences to detect demographic fluctuations applying several neutrality tests, such as R_2, Fu and Li's F*, Tajima's D, Fu's F_s and MAE. These tests are based on the assumption that most mutations that contribute to the genetic variation in natural populations are neutral (Kimura 1983). Y-chromosome markers were used to compare paternal and maternal genetic diversity in the surveyed populations.

In this study we compared the performance of different analytical methods to detect the existence of past demographic bottlenecks in two European grey wolf (*Canis lupus*) populations. The two populations selected were from the Iberian Peninsula (Spain and Portugal) and European Russia. Ecologically different, the Iberian wolf population is isolated and has suffered a single main demographic bottleneck in the second half of the twentieth century, whereas the European Russia wolf population has never been isolated and has experienced several demographic fluctuations during the last century (Aspi et al. 2009). We quantified and compared the levels of genetic variability of both populations using microsatellite, mitochondrial and Y-chromosome molecular markers and check the performance and consistency of bottleneck tests. Both bottleneck events may have been similar in relative intensity but since the Iberian population has a lower effective population size and is more isolated, we expected stronger evidence for the population decline.

Historic context of the wolf populations

The largest wolf population in Western Europe is located in the Iberian Peninsula (Boitani 2003), and it has been isolated from other European wolf populations at least since the end of the nineteenth century, when wolves from France and Central Europe disappeared (Valière et al. 2003). At the beginning of the twentieth century, Iberian wolves were distributed throughout the Peninsula. However, in the middle of the century, after wolf eradication campaigns using mainly poison, the population disappeared over most of its former range (Valverde 1971). In the early 1970s, the population was reduced to an all-time low, roughly estimated to be about 700 individuals (Grande del Brío 1984). Nevertheless, new popular awareness and conservation policies in the 1970s brought legal protection for the wolves in Spain (IUCN 1973), increasing their range and population size. The Iberian wolves, confined mainly to the Northwest of the Peninsula in the 1980s, expanded over more than 100,000 km^2 reaching, at the beginning of the twenty-first century, 254-322 packs and over 2,000 individuals (Álvares et al. 2005).

On the other hand, the wolf is an abundant predator in the Russian Federation (Bibikov et al. 1983). Wolves were never protected but neither were they endangered in this region (Bibikov 1994). Their occurrence was continuous over an enormous area, likely due to high density of natural prey and wide and dense boreal coniferous forests. Also, economic and political disorders coupled with weak wolf control programs (mainly due to the size of the wolf range) facilitated their prevalence (Bibikov 1994). During the twentieth century, three demographic peaks have been reported for wolves in the previous Soviet Union. The first peak was recorded in 1946, with an estimate of more than 40,000 individuals (Gubar 1996). After World War II an efficient wolf eradication campaign started and between 1960 and 1970 the population of wolves decreased to an all-time low (Bibikov et al. 1983). The second peak occurred in the 1980s, reaching approximately the same

Fig. 1 Wolf range distribution in Eurasia according to the IUCN (www.iucnredlist.org), and sampling regions

number as in the 1940s (Gubar 1996). However, at the beginning of the 1990s the number of wolves decreased to 22,000 individuals due to a new centralized extermination campaign (Ovsyanikov et al. 1998). From that point the number of wolves rose steadily, reaching more than 45,000 individuals at the end of the 1990s. Today, the largest wolf populations are distributed throughout the Northwestern and North-Caucasian regions of European Russia (Lomanov et al. 2000). In fact, European Russia contains a larger number of wolves than Asian Russia (Bibikov 1994; Lomanov et al. 2000). In this study, we have chosen four neighboring regions from European Russia, where the number of wolves at the time of sampling was about 1,843 individuals (Borisov et al. 1992; Lomanov et al. 1995, 2000).

Materials and methods

Samples

We analyzed 47 wolf samples from four geographical connected autonomous regions in Northwestern Spain, adding up to about 139,723 Km^2 (Asturias: 6 samples; Cantabria: 4; Galicia: 2 and Castilla y León: 35), and 47 from four geographical connected regions in Northern European Russia, covering about 308,281 km^2 (Kaluzshkaya: 2; Smolenskaya: 12; Vologodskaya: 5 and Tverskaya: 28) (Fig. 1). Assuming that the mean generation time for wolves is 3.4 years (Aspi et al. 2006), the Iberian wolf samples were collected about 7–11 generations after the main bottleneck event (from 1994 to 2008) whereas Russian samples were collected approximately 4–8 generations after the main event (from 1984 to 1996). The Iberian samples were obtained from carcasses sampled by local government personnel. The Russian samples corresponded to teeth from skulls deposited at the scientific collection of the Central Forest National Reserve (Zapovednik, Tverskaya region). While the Iberian samples represent an isolated population, the Russian samples represent a portion of a much larger wolf population range. However, here we have considered wolves from Russia as a unique population compared to the Iberian one (see below).

Laboratory procedures

DNA was extracted from tissue and tooth samples in separate rooms and on different days according to Sastre et al.

(2009). Blank DNA extractions and negative PCR controls were used throughout the study in order to detect exogenous DNA contamination. All PCR reactions were prepared under a laminar flow hood. A 333 base pairs (bp) DNA fragment of the 5′ end of the mitochondrial control region was amplified using primers LoboMit-F 5′-CT CCACCATCAGCACCCAAAG-3′ and LoboMit-R 5′-GT AACCCCCACGTTAGTATG-3′ as described by Ramírez et al. (2006). PCR products were sequenced with BIG Dye™ Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Sequences were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, MA, USA) and separated on an ABI PRISM 3730 automated sequencer (Applied Biosystems) according to the protocol provided by the manufacturer. All sequences were examined with SEQSCAPE 2.1.1 (Applied Biosystems).

Five canine primer pairs, MS34A, MS34B, MS41A, MS41B, 990 (Sundqvist et al. 2001; Bannasch et al. 2005), were used to amplify Y chromosome microsatellites from male wolf samples. The sex of the wolves was registered from all carcasses by field personnel. DNA from tooth samples was amplified in a PCR mixture containing 30–40 ng of DNA, PCR buffer (1×), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 1 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). In order to avoid typing errors in tooth DNA samples—with DNA of low quality and quantity—each sample was amplified four times (Sastre et al. 2009). DNA from blood samples was amplified in two multiplex PCR reactions with three (MS34A, MS41A and 990) and two markers (MS34B, MS41B). One primer from each pair was fluorescently labeled with 6-FAM, TET or HEX. All amplifications were carried out in 25-μl reactions. The thermal cycling profile included 10 min at 94°C followed by 40 cycles of 94°C (30 s), 55°C (or 58°C for MS34A, MS34B) (30 s) and 72°C (30 s), and completed with 10 min at 72°C. For the multiplex PCR reactions the annealing temperature was 57°C. Two microliters of PCR amplification product were added to a 10 μl mix of Formamide and ROX 70-500 (10:0.4) (BioVentures, Murfreesboro, TN, USA) and were run on an ABI PRISM 3730 and automatically sized according to the internal size standard. Microsatellite patterns were examined with GENEMAPPER 4.0 (Applied Biosystems).

We genotyped 13 unlinked autosomal microsatellite loci including eight dinucleotide (CPH5, CPH9 CXX366, CXX403, CXX410, CXX442, CXX459, CXX474) and five tetranucleotide markers (CXX2001, CXX2010, CXX2130, CXX2054, CXX2158) (Fredholm and Wintero 1995; Ostrander et al. 1995; Francisco et al. 1996). Multiplex PCR reactions for DNA from blood and tissue samples were performed as described elsewhere (Ramírez et al. 2006). Conditions for PCR reactions for tooth samples were the same as for blood samples but without multiplexing and repeating each amplification four times. As for Y-chromosome markers, 2 μl of PCR amplification product were added to a 10 μl mix of Formamide and ROX 70-500 (10:0.4) and genotyped on an ABI PRISM 3730. The determination of allele lengths was performed with GENEMAPPER 4.0.

Data analysis

Genetic diversity statistics for mtDNA sequences, such as number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences (K), as well as genetic differentiation (F_{ST}) (Hudson et al. 1992), were estimated using DNASP 4.10 (Rozas et al. 2003). Several neutrality statistics, often used to investigate demographic changes, were estimated by using a coalescent-based approach in DNASP (Ramos-Onsins and Rozas 2002). These neutrality tests were based on the distribution of mutation frequencies (Class I; R_2, Fu and Li's F* and Tajima's D), on the distribution of haplotype frequencies (Class II; Fu's F_s test statistic) and on the mean absolute error between the observed and the theoretical mismatch distribution (Class III; MAE) (Ramos-Onsins and Rozas 2002).

Genetic diversity for Y-chromosome microsatellites was estimated using GENALEX 6.1 (Peakall and Smouse 2006). Phylogenetic relationships between mitochondrial and Y-chromosome haplotypes were assessed by median-joining networks as implemented in NETWORK 4.5 (Bandelt et al. 1999). A maximum parsimony post-processing criterium was applied setting equal weight to each locus (Koblmüller et al. 2009).

Genotyping errors for autosomal microsatellites due to allelic dropout, false alleles and null alleles, were checked using MICRO-CHECKER 2.2 (van Oosterhout et al. 2004). Genetic variability statistics, such as number of alleles (A), allelic richness (Rs) and observed (Ho) and expected (H_E) heterozygosities, were estimated per locus and per population using FSTAT 2.9.3.2 (Goudet 2000). Deviations from Hardy–Weinberg equilibrium (HWE) were estimated from the inbreeding estimator F_{IS} (Guo and Thompson 1992) for each locus and population using ARLEQUIN 3.01 (Schneider et al. 2006), with Markov chains of 100,000 steps following 10,000 dememorization steps. Confidence interval values (95% CI) for F_{IS} were calculated using GENETIX 4.05.2 (Belkhir et al. 1999). Differences in genetic variability between the two populations were evaluated using Wilcoxon *W* signed-rank tests. To test for genetic differentiation between and within populations, pairwise F_{ST} values (Weir and Cockerham 1984)

were estimated using GENETIX. To visualize the distribution of genetic variation across individuals, we carried out a factorial correspondence analysis (FCA) (Benzecri 1973) as implemented in GENETIX, and a Bayesian model-based clustering approach in STRUCTURE 2.2 (Falush et al. 2007). Ignoring prior population information and using an admixture model with independent allele frequencies, STRUCTURE assigns individuals to populations. To assess the partition in a varying number of genetic clusters K, we run 100,000 Markov chain Monte Carlo repetitions and a burn-in period of 10,000 iterations for $K = 1$–5, repeated 20 times. Following Evanno et al. (2005), we chose the optimal K based on the rate of change of the log likelihood [ln Pr(X/K)] and its variance for $K = 1$–5. To estimate the effective population size (N_e) for the two populations we used a linkage approach, as implemented in the programs NEESTIMATOR (Peel et al. 2004) and LDN_e (Waples 2006).

Three common methods were applied to detect bottlenecks in the microsatellite data. The tests heterozygosity excess and mode-shift indicator were performed using BOTTLENECK 1.2.02 (Cornuet and Luikart 1996). Three mutation models (IAM, TPM and SMM) could be applied to simulate the distribution of alleles and from this estimate expected heterozygosity, being the two-phase mutation model (TPM) the most appropriate for microsatellite data provided that one-step mutations (SMM) predominate over multi-step changes (IAM) (Luikart et al. 1998). We tested for bottleneck signatures under the TPM model assuming p_g (proportion of multi-step mutations) = 0.10, σ^2 (variance) = 12, and using the one-tailed Wilcoxon's and sign tests to check for significance of the heterozygosity excess (Luikart and Cornuet 1997). The third method, M-ratio, was assessed using the software M_P_Val for each locus and averaging the value of M over loci (Garza and Williamson 2001). The significance of M was determined by comparing to a critical value (Mc), calculated from hypothetical populations in mutation-drift equilibrium. The statistic M suggests that a population has suffered a reduction in size when $M < Mc$. The Mc thresholds were generated using CRITICAL_M (Garza and Williamson 2001). We used the parameterization suggested by Garza and Williamson (2001) for the TPM model, where $p_g = 0.10$, Δ_g (average size of multi-step mutations) = 3.5, and μ (microsatellite mutation rate) = 5.0×10^{-4}/locus/generation. The threshold values are also dependent on θ, which depends on Ne according to the expression $\theta = 4\ N_e\mu$. As N_e per population was initially unknown, we calculated the Mc for different values of θ resulting from varying N_e from 50 to 3000 (minimum and maximum N_e expected per population; see estimates below) and obtained a range for θ of 0.1–6.

Results

mtDNA sequences

We identified a total of 6 haplotypes in the Russian population, all of them described in previous studies; W6, W14 (Vilà et al. 1999), W11 (Valière et al. 2003), W2, W5 and W21 (Pilot et al. 2006). We also found three (lu1, lu2, lu4) out of four haplotypes previously reported in Iberian wolves (Vilà et al. 1999) (Fig. 2a). Only haplotype lu3, identified by Vilà et al. (1999) in one individual from Portugal was not found among our samples.

Haplotype diversity and number of polymorphic sites were larger in Russian wolves than in their Iberian counterparts. Despite this, just one haplotype was predominant in each population, W6 in Russia (present in 45% of the samples) and lu1 in Spain (55%) (Table 1; Fig. 2a). Genetic differentiation between populations was highly significant ($F_{ST} = 0.396$; $P < 0.001$) due to the fact that they did not share haplotypes. However, the minimum spanning network showed low level of sequence divergence between populations and the haplotypes observed in each population did not form separate clusters (Fig. 2a).

The mismatch distribution of pairwise differences showed similar distribution patterns in both populations, failing to indicate population expansion (data not shown). Neutrality tests were not significant for the Russian population ($P > 0.05$ in all cases) failing to indicate a population reduction. In contrast, in the Iberian population, class I and II neutrality tests were significant ($P < 0.05$), suggesting a decline in population size due to their positive value, while the test based on the mismatch distribution was not significant (MAE; $P > 0.05$) (Table 1).

Y-chromosome microsatellites

We identified 10 haplotypes among Russian wolves and 4 haplotypes among Iberian wolves (Table 2). No haplotypes were shared between the populations. Haplotype YIW02 was present in half (50%) of the individuals tested in the Iberian Peninsula. As for mtDNA data, Y-chromosomal diversity was higher in Russian wolves than in Iberian ones (Table 2), and, as for the mtDNA, the minimum median-joining network did not reveal a separation between populations (Fig. 2b).

Autosomal microsatellites: preliminary analyses

The program MICRO-CHECKER identified 3 loci with heterozygosity deficiency ($P < 0.01$) in the Russian (CXX 366, CXX2130 and CXX2158) and Iberian (CXX2001,

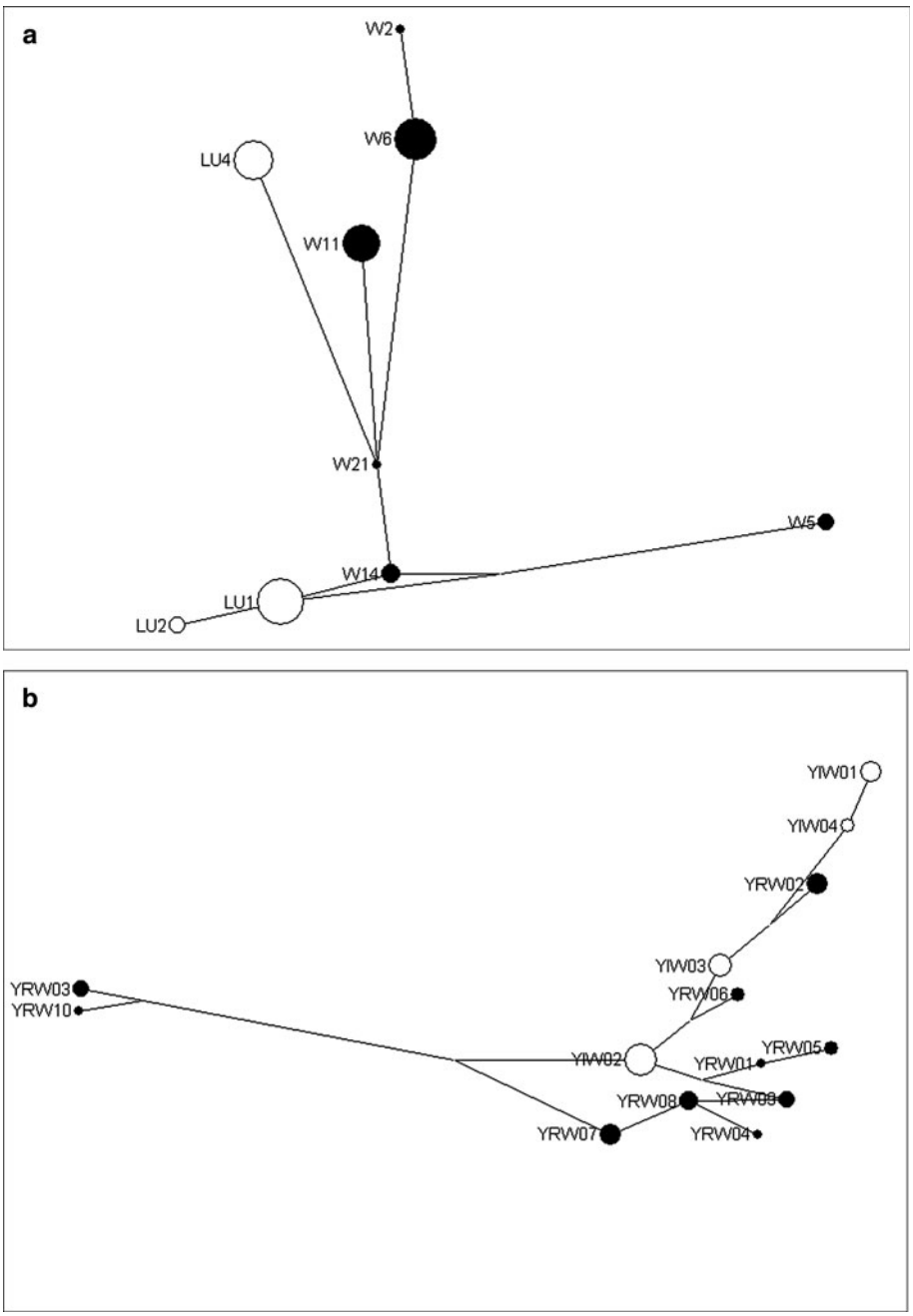

Fig. 2 Median-Joining network of (**a**) mtDNA and (**b**) Y-chromosome haplotypes in Russian (*black circles*) and Iberian (*white circles*) wolves. The size of the *circles* is proportional to the haplotype frequency, and branch length is proportional to the number of nucleotides differences

Table 1 Mitochondrial diversity and neutrality tests for two European wolf populations

Mitochondrial DNA sequences										
	N	H	Hd	π	S	Class I			Class II	Class III
						R_2	F*	D	F_s	MAE
Russian wolf	47	6	0.672 ± 0.043	0.016	10	0.157	1.073	1.151	2.932	0.905
Iberian wolf	47	3	0.555 ± 0.041	0.010	6	0.211*	1.770*	2.236*	6.061*	1.083

N sample size, *H* haplotype number, *Hd* haplotype diversity, π nucleotide diversity, *S* number of polymorphic sites

* $P < 0.05$

Table 2 Y chromosome haplotypes and diversity in Russian and Iberian wolves for five microsatellite loci

Haplotype	MS34A	MS34B	MS41A	MS41B	990	Frequency (%)
Russian wolves						
YRW01	181	179	207	222	126	3.7
YRW02	175	179	207	228	126	18.5
YRW03	173	183	207	216	130	11.1
YRW04	181	177	207	218	124	3.7
YRW05	181	179	209	222	126	7.4
YRW06	177	179	209	224	126	7.4
YRW07	179	177	207	216	124	18.5
YRW08	179	177	207	218	124	14.9
YRW09	179	177	207	220	124	11.1
YRW10	173	181	207	214	130	3.7
Where N = 27; N_a = 12; H = 10; Hd = 0.602 ± 0.095						
Iberian wolves						
YIW01	175	177	207	224	124	19.2
YIW02	177	179	207	222	126	50.0
YIW03	177	179	207	226	126	23.1
YIW04	175	177	207	226	124	7.7
Where N = 26; N_a = 1; H = 4; Hd = 0.360 ± 0.100						

N sample size, N_a number of unique alleles per population, *H* haplotype number, *Hd* haplotype diversity

Alleles are represented by their sizes

CXX403 and CXX2158) populations, suggesting the presence of null alleles in these loci. Null alleles can be detected as an excess of homozygotes leading loci to deviations from HWE (Carlsson 2008). The heterozygosity excess test requires molecular markers to be in HWE (Busch et al. 2007). For that reason, we discarded the locus CXX2158 from further analyses because it deviated significantly from HWE in both populations ($P < 0.001$). Moreover, to evaluate the impact of including the markers that show significant deviation from equilibrium per population, bottleneck tests were repeated including and excluding these markers (see Table 4).

Population structure and effective population size

F_{ST} showed significant differentiation between the two wolf populations ($F_{ST} = 0.131$; $P < 0.05$). The factorial correspondence analysis also indicated that the Iberian wolves were distinct from Russian wolves on the first factorial axis, FA-I, explaining 7.86% of the total genetic diversity (Figure not shown). In the same way, the Bayesian model-based clustering method showed a peak in ΔK (Fig. 3a, b; Evanno et al. 2005) for K = 2, which separated in two clear clusters Russian and Iberian wolves (Fig. 3c). F_{ST} values among the four Iberian (F_{ST} = 0.049) and Russian regions (F_{ST} = 0.037) were very low indicating very weak and not significant substructure within populations.

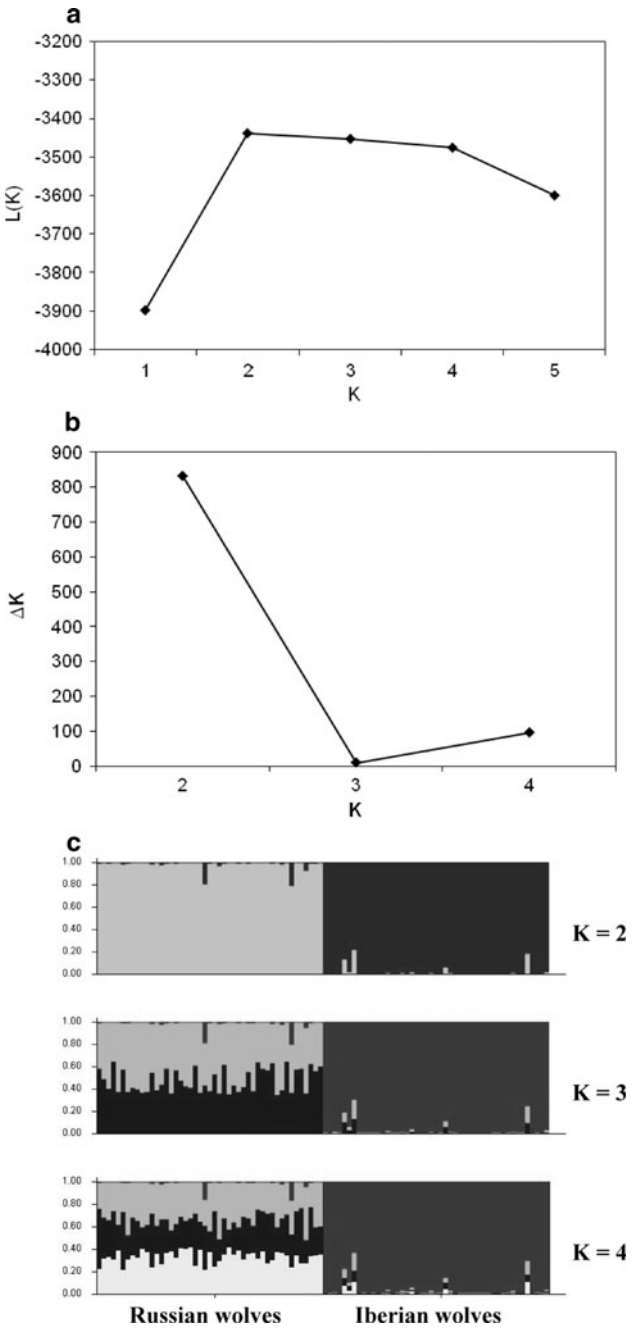

Fig. 3 Bayesian clustering approach for two wolf populations. **a** Mean likelihood L(K) over 20 runs assuming K clusters (K = 1–5). **b** ΔK following Evano et al. 2005, where the modal value of the distribution is the highest level of structuring. **c** Individual assignment using K = 2, K = 3 and K = 4 clusters. Each individual is represented as a vertical bar sectioned into K *colored segments*, whose length is proportional to the likelihood of assignment to the K cluster

Genetic diversity was significantly higher in Russian wolves than in Iberian wolves (A, R_s, H_E; *W* tests, $P < 0.05$ in all cases). Both populations showed $H_E > H_O$ at almost

Table 3 Genetic diversity for each microsatellite in two wolf populations

Locus	Russian wolf ($n = 47$)						Iberian wolf ($n = 47$)					
	A	N_a	Rs	H_O	H_E	F_{IS} (95% CI)	A	N_a	Rs	H_O	H_E	F_{IS} (95% CI)
CXX2001	7	2	6.936	0.702	0.749	0.051	6	1	6.000	0.468	0.784	0.401***
CXX2010	8	5	7.745	0.745	0.729	−0.034	3	0	2.997	0.574	0.512	−0.124
CXX2054	14	8	13.992	0.638	0.819	0.222***	7	1	6.936	0.447	0.552	0.166
CXX403	10	5	9.809	0.702	0.864	0.179*	5	0	4.997	0.277	0.630	0.549***
CXX410	9	4	8.809	0.787	0.820	0.034	5	0	5.000	0.489	0.747	0.341***
CXX442	4	2	4.000	0.511	0.664	0.225	3	1	3.000	0.467	0.407	−0.148
CPH5	6	2	5.872	0.553	0.658	0.161	7	3	7.000	0.689	0.747	0.078**
CXX366	7	4	6.933	0.468	0.755	0.372***	4	1	4.000	0.477	0.539	0.116
CPH9	13	7	13.000	0.841	0.870	0.033***	6	0	6.000	0.636	0.766	0.156*
CXX2130	12	6	11.910	0.717	0.875	0.180	8	2	7.913	0.652	0.754	0.132*
CXX474	8	5	7.929	0.617	0.786	0.215*	4	1	3.936	0.468	0.488	0.011
CXX459	9	2	8.954	0.674	0.793	0.138	8	1	7.933	0.660	0.810	0.182***
Total	8.9	52	8.824	0.663	0.782	0.147 (0.07–0.20)*	5.5	11	5.476	0.525	0.645	0.177 (0.10–0.23)*

Deviations from Hardy–Weinberg equilibrium were assessed from the fixation index F_{IS} for each locus

A allele number, N_a number of unique alleles per locus and population, *Rs* allelic richness ($n = 44$), H_O observed heterozygosity, H_E expected heterozygosity

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

all loci, and positive F_{IS} values (Table 3), not significantly different between populations (*W* test, $P > 0.05$). Finally, the two programs based on the linkage disequilibrium among the 12 microsatellite loci gave similar estimates of N_e for the Iberian population (NEESTIMATOR, $N_e = 53.8$ [95% confidence interval: 41.4–73.8]; LDN_e, $N_e = 43.2$ [29.9–68.5]) but less similar for the Russian population (NEESTIMATOR, $N_e = 312.5$ [167.1–1741.6]; LDN_e, $N_e = 138.0$ [75.9–490.4]).

Bottleneck tests

The Wilcoxon's test for heterozygosity excess was significant for the Russian population ($P = 0.026$) (Table 4). The Iberian wolves also showed heterozygosity excess at the majority of loci but the sign and Wilcoxon tests were not significant ($P > 0.05$) (Table 4). However, population contraction in Spain was supported by the mode-shift indicator (Table 4; Fig. 4). A loss of rare alleles was detected because the proportion of rare alleles ($q < 0.05$) was 24.4% while the proportion of the third allele class ($0.1 < q < 0.2$) was 26.8% (also higher than the frequency of alleles of the class [$0.05 < q < 0.1$]). Thus, we inferred that the Iberian population was not at mutation-drift equilibrium. By contrast, the allelic frequency distribution for the Russian population was more L-shaped, as expected for populations that have not gone through recent bottlenecks (Fig. 4).

The *M*-ratio for Iberian samples was 0.615, indicating that numerous alleles were absent from the population relative to the total number expected under the TPM model (Table 4). *M* was significantly below *Mc* over a wide range of N_e values (from $N_e = 50$ to 3,000), consistently indicating the occurrence of a bottleneck. The *M*-ratio for Russian samples was 0.715, always slightly below *Mc*-critical values although above the threshold of 0.68 suggested by (Garza and Williamson 2001) to safely infer past bottlenecks.

To assess if the results were influenced by having included two loci that are not in HW equilibrium within each population, the analyses were repeated just including 10 microsatellites that did not appear to have significant deviations from HWE. The results using 10 or 12 microsatellite loci were similar (Table 4). Therefore, we agree with Carlsson (2008) that the presence of null alleles seems not affect the tests performed.

Discussion

Mitochondrial and Y-chromosome variation were higher in the non-isolated Russian population than in the isolated Iberian population (Tables 1, 2). However, the variability found in Iberian wolves was about two-fold compared to isolated and genetically depauperate populations described in previous studies. Pilot et al. (2006) found that wolf populations from Eastern Europe had multiple mtDNA haplotypes which were widely distributed, whereas wolves from Italy, isolated like Iberian wolves, had a unique haplotype (Randi et al. 2000). We observed three haplotypes in the Iberian Peninsula, lu1 and lu2 haplotypes not

Table 4 Bottleneck detection tests in two wolf populations using a mutation rate of 5.0×10^{-4}

Test (12 microsatellite loci)	Parameters			Russian wolves ($n = 47$)	Iberian wolves ($n = 47$)
Heterozygosity excess	$\sigma^2 = 12$	$p_g = 0.10$	Sign test	10:2 ($P = 0.06$)[a]	9:3 ($P = 0.163$)
			Wilcoxon	$P = 0.026$	$P = 0.088$
M-ratio	$\Delta_g = 3.5$	$p_g = 0.10$		M = 0.715	M = 0.615
	$\theta = 0.1$, $N_e = 50$		$M_c = 0.85$	$P = 0.0000$[b]	$P = 0.0000$
	$\theta = 0.6$, $N_e = 300$		$M_c = 0.81$	$P = 0.0026$	$P = 0.0000$
	$\theta = 1.0$, $N_e = 500$		$M_c = 0.79$	$P = 0.0029$	$P = 0.0000$
	$\theta = 6.0$, $N_e = 3000$		$M_c = 0.72$	$P = 0.0422$	$P = 0.0001$
Mode-shift				Normal L-shaped distribution	Shifted mode
Test (10 microsatellite loci)[c]					
Heterozygosity excess	$\sigma^2 = 12$	$p_g = 0.10$	Sign test	9:1 ($P = 0.032$)	7:3 ($P = 0.294$)
			Wilcoxon	$P = 0.042$	$P = 0.161$
M-ratio	$\Delta_g = 3.5$	$p_g = 0.10$		M = 0.710	M = 0.594
	$\theta = 0.1$, $N_e = 50$		$M_c = 0.84$	$P = 0.0004$	$P = 0.0000$
	$\theta = 0.6$, $N_e = 300$		$M_c = 0.80$	$P = 0.0023$	$P = 0.0000$
	$\theta = 1.0$, $N_e = 500$		$M_c = 0.78$	$P = 0.0049$	$P = 0.0000$
Mode-shift				Normal L-shaped distribution	Shifted mode

σ^2 Variance, p_g proportion of multi-step mutation (IAM), Δ_g average size of multi-step mutations, M_c critical value

[a] Heterozygosity excess versus heterozygosity deficit loci

[b] P = probability values for a greater M ratio

[c] CPH9 and CXX366 loci and CXX2001 and CXX403 loci deviated significantly from HWE in the Russian and Iberian populations respectively and were discarded from the second part of the analysis

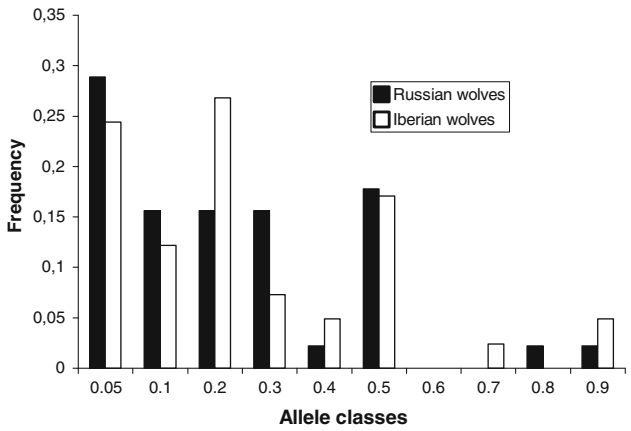

Fig. 4 Frequency distribution of allele classes for Iberian and Russian wolves

being observed elsewhere in Europe (Valière et al. 2003). In the same way, Sundqvist et al. (2001) reported 9–10 Y-chromosome haplotypes among Eastern wolf populations and only 2 haplotypes in Scandinavian wolves, which derived from a founding event involving just two males (see also Vilà et al. 2003). For the Iberian wolf population, the number of Y chromosome haplotypes was intermediate (four haplotypes).

As for uniparental markers, autosomal microsatellite variability measures such as allelic diversity (A) and expected heterozygosity (H_E) were higher in Russian wolves (A = 8.9; $H_E = 0.78$) than in Iberian wolves (A = 5.5; $H_E = 0.65$), but both were close to values found in non-isolated wolf populations from North America (A = 5.9, $H_E = 0.74$, Carmichael et al. 2007; A = 7.9, $H_E = 0.67$, Musiani et al. 2007; A = 7.0, $H_E = 0.72$, Kobmüller et al. 2009) or northwestern Russia (A = 5.7, $H_E = 0.71$; A = 4.7, $H_E = 0.64$, Aspi et al. 2009), and higher than observed for isolated populations from Italy (A = 4.4, $H_E = 0.49$, Lucchini et al. 2004) or Scandinavia (A = 3.1, $H_E = 0.52$, Flagstad et al. 2003). Therefore, even though the genetic variability was lower in the isolated Iberian population, it does not seem extremely depleted of diversity. However, comparisons of diversity measures derived from different sets of markers can only be taken as a rough approximation.

The genetic evidence that a bottleneck occurred in the Iberian population was provided by both maternal and biparental markers: positive neutrality tests for mtDNA sequences and significant M-ratio and mode-shift tests for autosomal microsatellites. Only two tests failed to evidence the demographic decline in this isolated population; the neutrality test based on the mismatch distribution (MAE), likely because it is very conservative (Ramos-Onsins and Rozas 2002), and the heterozygosity excess test which compares observed heterozygosity with the expected value under the assumption that the population is at mutation-

drift equilibrium (Cornuet and Luikart 1996). As the allelic diversity is rapidly reduced, a heterozygosity excess can be observed following a bottleneck event. Several reasons could contribute to the lack of significance: presence of null alleles, inbreeding, population substructuring (Wahlund's effect) and low statistical power. Null alleles do not seem to explain the result since the same results were observed after excluding two loci that were not in HWE in each population (Table 4). With regard to inbreeding, this does not seem to be the reason for the results either: we found values of FIS that were high in both populations (0.147 and 0.177) and not significantly different (*W* test, $P > 0.05$), but the test was only significant for the Russian population. Fragmentation does not seem to explain the results either because the samples were collected over a relatively smaller (and continuous) area than for the Russian population. In the same way, lack of statistical power does not seem to be the reason since the number of samples typed falls within the range recommended by Luikart et al. (1998) to achieve high power in the test (between 15–40 individuals and between 10–15 microsatellites). However, it is possible that the lower number of alleles in the Iberian population (see Table 3) has reduced the power of the test in this population, or that the bottleneck is too recent to produce a detectable heterozygosity excess in this population (see Cornuet and Luikart 1996).

In the Russian population, we have detected the genetic signature of the bottleneck using autosomal data and despite the fact that migration and/or mutation could erase bottleneck signatures in few generations (Keller et al. 2001; Busch et al. 2007), but we failed to detect such signature using mtDNA. The four Russian regions sampled have ecological and physical continuity, the landscape not being fragmented by noticeable topographical barriers and thus apparently allowing high rates of gene flow. Since the sampling locations are within a much wider distribution, gene flow is also possible with neighbouring areas. We found excess of heterozygosity in the population regardless of using 10 or 12 microsatellite loci (Table 4). Furthermore, even though M (0.72) > 0.68, and thus a reduction in size cannot be confidently assumed (Garza and Williamson 2001), loss of alleles was evident because $M < Mc$ (Table 4). However, the distribution of allele frequencies was biased towards rare alleles (L-shaped), failing to show a large loss of rare alleles or indicating some recovery due to mutation or migration. In the same way, no genetic decline was evidenced using maternal inherited markers. Perhaps the effective population size in Russia is large enough to make difficult the identification of the bottleneck by using short mtDNA sequences as the ones used here.

Finally, official data showed that, from 1984 to 1995, the total number of wolves in Kaluzskaya (178), Smolenskaya (347), Volgodskaya (708) and Tverskaya (635) was about 1,843 individuals (Borisov et al. 1992; Lomanov et al. 1995, 2000). Frankham et al. (2002) estimated that, on average and across a very wide range of species, the mean value for N_e/N was 0.11. With this estimate, and considering the size of the wolf population in the studied region, we could expect N_e values around 203, similar to the values that we estimated (312.5 and 138.0). However, this is the value that should be expected if the wolf population was isolated from the population in the rest of Russia. If the region was widely connected by gene flow with the rest of the Russian population, with a total population estimated to be close to 40,000 wolves, we would expect a much larger effective population size. Consequently, our relatively low effective population size estimates are consistent with fragmentation (England et al. 2010). Similarly, the large value observed for F_{IS} indicates the existence of some degree of fragmentation within the study area. More detailed population genetic studies including samples from the entire range of wolves in Russia would allow identifying discontinuities and proper units for management.

In the case of the Iberian population, the estimated effective population size is about 50 (two estimates, 53.8 and 43.2), which is about 2.5% of the mentioned estimate of 2,000 individuals, well below the 11% ($N_e/N = 0.11$) estimated by Frankham et al. (2002). In this case, our sample can be considered to represent the entire distribution range of the wolf in the Spain (except for two very small and isolated nuclei in the South and in the North East; Álvares et al. 2005, Sastre et al. 2007) and the small effective population size cannot be interpreted as a result of a biased distribution of the samples (not representing the entire population). This could indicate that the population size has been overestimated (for example, by including juveniles; Vilà 2010) and/or the genetic diversity has been more dramatically reduced by the bottleneck than initially suspected.

To summarize, we have detected demographic bottlenecks in both populations regardless of the high growth rate of the wolf populations (the Iberian population could have experienced a two-to three-fold increase in 30 years). Maternal markers such as mtDNA sequences were effective detecting the demographic decline in the small isolated population but not in the large one. In contrast, biparental markers were effective showing the effects of the bottleneck in both populations, the *M*-ratio test being useful for both of them. However, due to the different results obtained with the tests, we recommend carrying out multiple approaches, also using different kinds of genetic markers, in order to detect bottlenecks. Furthermore, given the small effective population size estimated for the Iberian population, we suggest continuous surveillance because its isolation can lead to a fast loss of genetic diversity and

adaptive potential. Any fragmentation within this population would further accelerate this loss. On the other hand, the effective population size for Russian wolves could indicate that some fragmentation exists indicating that the entire Russian wolf population should not be seen as a unique management unit.

Acknowledgments Samples were moved from the Russian Federation to Spain according to CITES and VETER requirements. Animals were not killed for the purpose of this manuscript. We are grateful to the personnel from the Central Forest National Reserve (Zapovednik, Russia), especially to Pavel Koravlov, for supplying wolf tooth samples. We thank all the personnel from the "Consejería de Medio Ambiente, Junta de Castilla y León", especially to Agustín Noriega, for supplying wolf tissue samples. Thanks are due also to Sebastian Ramos-Onsins and Joaquim Casellas for valuable comments on the analyses, to Miki Monguilod for IT support, to Gary Walker for linguistic revision and two anonymous reviewers for helpful comments on the manuscript. Carles Vilà work was supported by the "Programa para la Captación del Conocimiento para Andalucía" (Andalusian Government, Spain). Financial support was provided by the "Servei Veterinari de Genètica Molecular" (SVGM).

References

Álvares FI, Barroso JC, Blanco J et al. (2005) Wolf status and conservation in the Iberian Peninsula. International Congress Frontiers of Wolf Recovery. Colorado Springs, Colorado, October 2005

Aspi J, Roininen M, Ruokonen M, Kojola I, Vilà C (2006) Genetic diversity, population structure, effective population size and demographic history of the Finnish wolf population. Mol Ecol 15:1561–1579

Aspi J, Roininen M, Kiiskilä J, Ruokonen M, Kojola I, Bljudnik L, Danilov P, Heikkinen S, Pulliainen E (2009) Genetic structure of the northwestern Russian wolf populations and gene flow between Russia and Finland. Conserv Genet 10:815–826

Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Bannasch DL, Bannasch MJ, Ryun JR, Famula TR, Pedersen NC (2005) Y chromosome haplotype analysis in purebred dogs. Mamm Genome 16:273–280

Belkhir K, Borsa P, Goudet J, Chikhi L, Bonhomme F (1999) GENETIX, Logiciel sous Windows TM pour la Génétique des Populations. Laboratoire Génôme et Populations, CNRS UPR 9060. Université de Montpellier II, Montpellier, France

Benzecri J (1973) L'Analyse des Données. Tome I: la Taxinomie; Tome II: l'Analyse des Correspondances. Dunod Press, Paris

Bibikov DI (1994) Wolf problem in Russia. Lutreola 3:10–14

Bibikov DI, Ovsyannikov NG, Filimonov AN (1983) The status and management of the wolf population in the USSR. Acta Zoologica Fennica 174:269–271

Boitani L (2003) Wolf conservation and recovery. In: Mech LD, Boitani L (eds) Wolves. Behavior, ecology and conservation. University of Chicago Press, Chicago, pp 317–340

Borisov BP, Gibet LA, Gubar JP et al. (1992) Senior management of the hunting facilities economy at RSFSR. The central research laboratory of the hunting facilities economy and reserves. Moscow (in Russian)

Busch JD, Waser PM, DeWoody JA (2007) Recent demographic bottlenecks are not accompanied by a genetic signature in banner-tailed kangaroo rats (*Dipodomys spectabilis*). Mol Ecol 16:2450–2462

Carlsson J (2008) Effects of microsatellite null alleles on assignment testing. J Hered 99:616–623

Carmichael LE, Krizan J, Nagy JA, Fuglei E, Dumond M, Johnson D, Veitch A, Berteaux D, Strobeck C (2007) Historical and ecological determinants of genetic structure in artic canids. Mol Ecol 16:3466–3483

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

England PR, Luikart G, Waples RS (2010) Early detection of population fragmentation using linkage disequilibrium estimation of effective population size. Conserv Genet 11:2425–2430

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14:2611–2620

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Fay JC, Wu CI (1999) A human population bottleneck can account for the discordance between patterns of mitochondrial versus nuclear DNA variation. Mol Biol Evol 16:1003–1005

Flagstad ∅, Walker CW, Vilà C, Sundqvist AK, Fernholm B, Hufthammer AK, Wiig ∅, Koyola I, Ellegren H (2003) Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. Mol Ecol 12:869–880

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

Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge

Fredholm M, Wintero AK (1995) Variation of short tandem repeats within and between species belonging to the Canidae family. Mamm Genome 6:11–18

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. Mol Ecol 10:305–318

Goudet J (2000) FSTAT version 2.9.3.2. Computer package for PCs. Institute of Ecology, Lausane, Switzerland

Grande del Brío R (1984) El lobo Ibérico Biología y mitología. Editorial Hermann Blume, Madrid

Gubar JP (1996) The wolf in Russia caught between hunters and environmentalists. Russ Conserv News 8:19–20

Guo S, Thompson E (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. Genetics 132(2):583–589

International Union for Conservation of Nature (IUCN) (1973) Red list of threatened species. http://www.iucnredlist.org/

Keller LF, Jeffery KJ, Arcese P et al (2001) Immigration and the ephemerality of a natural population bottleneck: evidence from molecular markers. Proc R Soc Lond 268:1387–1394

Kimura M (1983) The neutral theory of molecular evolution. Cambridge University Press, Cambridge, UK

Koblmüller S, Nord M, Wayne RK, Leonard JA (2009) Origin and status of the Great Lakes wolf. Mol Ecol 18:2313–2326

Lomanov IK, Borisov BP, Bolodnina OA et al (1995) The hunting animals of Russia. Department on protection and rational use. State service of the account of the hunting resources, Moscow (in Russian)

Lomanov IK, Borisov BP, Bolodnina OA et al (2000) The hunting animals of Russia. State service of the account of the hunting resources. Ministry of agriculture of the Russian Federation, Moscow (in Russian)

Lucchini V, Galov A, Randi E (2004) Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. Mol Ecol 13:523–536

Luikart G, Cornuet JM (1997) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conserv Biol 12:228–237

Luikart G, Allendorf FW, Cornuet JM, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered 89:238–247

Musiani M, Leonard JA, Cluff HD, Gates CC, Mariani S, Paquet PC, Vilà C, Wayne RK (2007) Differentiation of tundra/taiga and boreal coniferous forest wolves: genetics, coat colour and association with migratory caribou. Mol Ecol 16:4149–4170

Ostrander EA, Mapa FA, Yee M, Rine J (1995) One hundred and one simple sequence repeats-based markers for the canine genome. Mamm Genome 6:192–195

Ovsyanikov N, Bibikov DI, Bologov VV (1998) Battling with wolves: Russia's decades-old struggle to manage its fluctuating wolf population. Int Wolf Center Publ 8(1):16–19

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Peel D, Ovenden JR, Peel SL (2004) NeEstimator: software for estimating effective population size, version 1.3. Queensland Government, Department of Primary Industries and Fisheries, Australia

Pilot M, Jedrzejewski W, Branicki W et al (2006) Ecological factors influence population genetic structure of European grey wolves. Mol Ecol 15:4533–4553

Ramírez O, Altet L, Enseñat C, Vilà C, Sánchez A, Ruiz A (2006) Genetic assessment of the Iberian wolf *Canis lupus signatus* captive breeding program. Conserv Genet 7:861–878

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. Mol Biol Evol 19(12):2092–2100

Randi E, Lucchini V, Christensen MF et al (2000) Mitochondrial DNA variability in Italian and East European wolves: detecting the consequences of small population size and hybridization. Conserv Biol 14:464–473

Reed DH, Briscoe DA, Frankham R (2002) Inbreeding and extinction: the effect of environmental stress and lineage. Conserv Genet 3:301–307

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DNASP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497

Saccheri IJ, Wilson IJ, Nichols RA, Bruford MW, Brakefield PM (1999) Inbreeding of bottlenecked butterfly populations: estimation using the likelihood of changes in marker allele frequencies. Genetics 151:1053–1063

Sastre N, Francino O, Lampreave G et al. (2007) Seguimiento del lobo (*Canis lupus*) en Cataluña mediante el análisis genético de muestras no invasivas. In: XXXVI Congreso de la Sociedad Española de Genética. SEG 2007, León, p 127

Sastre N, Francino O, Lampreave G et al (2009) Sex identification of wolf (*Canis lupus*) using non-invasive simples. Conserv Genet 10:555–558

Schneider S, Roessli D, Excoffier L (2006) Arlequin ver 3.01: An integrated software package for population genetics data analysis. Computational and Molecular Population Genetics Lab, University of Berne, Switzerland

Sundqvist AK, Ellegren H, Olivier M, Vilà C (2001) Y chromosome haplotyping in Scandinavian wolves (*Canis lupus*) based on microsatellite markers. Mol Ecol 10:1959–1966

Valière N, Fumagalli L, Gielly L et al (2003) Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. Anim Conserv 6:83–92

Valverde JA (1971) El lobo español. Montes 159:228–241

Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Vilà C (2010) Viabilidad de las poblaciones ibéricas de lobos. Enseñanzas de la genética para la conservación. In: Fernández-Gil A, Álvares F, Vilà C, Ordiz A (eds) Los lobos de la Península Ibérica. Propuestas para el diagnóstico de sus poblaciones. ASCEL, Palencia, Spain, pp 157–171

Vilà C, Amorim IR, Leonard JA et al (1999) Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. Mol Ecol 8:2089–2103

Vilà C, Sundqvist AK, Flagstad ∅ et al (2003) Rescue of a severely bottlenecked wolf *Canis lupus* population by a single immigrant. Proc R Soc 270:91–97

Waples R (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7:167–184

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370