Conservation Genetics **3:** 31–45, 2002.
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Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation

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Received 20 December 2000; accepted 6 May 2001

Key words: admixture analysis, assignment test, Bayesian clustering, *Canis lupus*, dog, wolf

Abstract

Hybridization with free-ranging dogs is thought to threat the genetic integrity of wolves in Europe, although available mtDNA data evidenced only sporadic cases of crossbreeding. Here we report results of population assignment and genetic admixture analyses in 107 wild-living Italian wolves, 95 dogs including 30 different breeds and feral dogs, and captive-reared wolves of unknown or hybrid origins, which were genotyped at 18 microsatellites. Two Italian wolves showed unusually dark coats ("black wolves"), and one showed a spur in both hindlegs ("fifth finger wolf"), suggesting hybridization. Italian wolves showed significant deficit of heterozygotes, positive F_{IS} values and deviations from Hardy-Weinberg equilibrium. Genetic variability was significantly partitioned between groups, suggesting that wolves and dogs represent distinct gene pools. Multivariate ordination of individual genotypes and clustering of inter-individual genetic distances split wolves and dogs into two different clusters congruent with the prior phenotypic classification, but hybrids and wolves of unknown origin were not identified from genetic information alone. By contrast, a Bayesian admixture analysis assigned all the Italian wolves and dogs to two different clusters, independent of any prior phenotypic information, and simultaneously detected the admixed gene composition of the hybrids, which were assigned to more than one cluster. Captive-reared wolves of unknown origin were prevalently assigned to the Italian wolf population. Admixture analyses showed that one "black wolf" had mixed ancestry in the dog gene pool and could be a hybrid, while the other two wolves with unusual phenotypes were assigned to the Italian wolf population.

Introduction

Wolf-like canids form a monophyletic clade of closely related species within the dog family Canidae (Wayne et al. 1997). They have identical karyotypes, can hybridize and produce fertile offspring in captivity and in nature where they overlap (Wayne et al. 1995). Risk of natural hybridization may be higher in areas where a species is locally rare and in sympatry with another overabundant species (i.e., wolves – *Canis lupus* – and coyotes – *C. latrans* – in Minnesota and eastern Canada; Lehman et al. 1991), or where wild canids are in contact with free-ranging domestic dogs, as it was documented for the Ethiopian wolf (*C. simensis*; Gottelli et al. 1994), and feared for some wolf populations in Europe (Butler 1994; Rhymer and Simberloff 1996). Hybridization has the potential to produce morphological, physiological and behavioral changes in captive and wild-living canids (Mengel 1971; Thurber and Peterson 1991; Lariviere and Crete 1993), and eventually led to the origin of a new taxon, the red wolf (*C. rufus*; Wayne et al. 1995). Therefore, hybridization and introgression of domestic genes can threaten the integrity of the gene pool of wild canids (Boitani 1984; Gottelli et al. 1994).

Direct human persecution and forest clearance lead wolves to disappear from most of Europe before the end of 19th century (Delibes 1990; Boitani 1995).

Isolated and declining populations persisted in Iberian Peninsula, Greece and in Italy, where less than 100 wolves survived in the central-southern Apennines during the 1970s (Zimen and Boitani 1975). Legal protection and the increase of wild ungulate populations efficiently contrasted the decline of wolves in Italy, which recently expanded and recolonized parts of their historical range in the north-western Apennines and western Alps (Breitenmoser 1998; Corsi et al. 1999). During the wolf population bottleneck the number of free-ranging dogs increased dramatically, thus raising the risk of hybridization (Boitani and Fabbri 1983). Nowadays, the Italian wolfs are estimated to number about 500, while the dogs which are at least partially free to range in rural areas of Italy could exceed 1,000,000 (Corsi et al. 1999; Genovesi and Dupré 2000).

Presence of hybrid wolves has been anecdotical reported in Europe and the near East (Mendelssohn 1982; Blanco et al. 1992), and directly observed at least once in Italy (Boitani 1983). However, extensive genetic analyses did not show any evidence of introgression of dog mitochondrial DNA (mtDNA) in Spanish and Italian wolves (Vilà and Wayne 1999; Randi et al. 2000), although a few putative hybrids were identified in Bulgaria (Randi et al. 2000), and Latvia (Andersone et al., submitted). The maternally inherited mtDNA is a highly diagnostic marker of hybridization, because wolf populations worldwide do not share haplotypes with any dogs typed so far (Okumura et al. 1996; Vilà et al. 1997; Vilà and Wayne 1999; Randi et al. 2000). These findings suggest that hybridization is uncommon or strictly directional, or that first generation crosses do not backcross into wolf populations (Lehman et al. 1991; Vilà and Wayne 1999; Randi et al. 2000).

Hybridization in canids was documented mainly by microsatellite genotyping (Roy et al. 1994). These biparental genetic markers are usually variable enough to allow for the unequivocal identification of all the sampled individuals in mammalian populations (Paetkau et al. 1998, and reference therein). Thus, individuals and not populations can be used as units for clustering procedures, such as multivariate ordination of individual scores (Sneath and Sokal 1973; She et al. 1987), or genetic distance-based approaches (Bowcock et al. 1994). These methods are simple and intuitive, but evaluating consistency and statistical significance of clusters, which must be identified visually, may be problematic. Therefore, these methods are more suited to exploratory data analysis than to precise statistical inference (Pritchard et al. 2000). More efficient methods include maximum likelihood assignment procedures (Paetkau et al. 1995; Rannala and Mountain 1997), and Bayesian clustering models (Pritchard et al. 2000). In these procedures, individual genotypes can be assigned to populations irrespective of whether or not their potential source populations are known. The origin of individuals can be determined by calculating the probability of each individual multilocus genotype in each population, assuming that the individual comes from that population. Cornuet et al. (1999) described also a distance-based method, which computes the average inter-individual distances from the population means, and assigns each individual to its closest population. Bayesian models (Pritchard et al. 2000) aim to infer the structure of a data set by assuming that observations from each sample are random draws from unknown gene frequency distributions, in which the marker loci are unlinked and at Hardy-Weinberg (HWE) and linkage (LE) equilibrium. Population structure within a data set is detected by the presence of Hardy-Weinberg and linkage disequilibrium, and is modelled by assuming that the genotype of each individual is a mixture drawn at random from a number of different populations. The number of contributing populations can be estimated and, for a given number of populations, their gene frequencies and the admixture proportions for each individual are all jointly estimated. In this way the sampled population is subdivided into a number of different subpopulations that effectively cluster the individuals. Then, individuals of *a-priori* known or unknown origin may be assigned probabilistically to the subpopulations.

In this study we analyse allelic variation at 18 canine microsatellite loci (Ostrander et al. 1993; Dolf et al. 2000) with the aims: (1) to estimate the extent of genetic differentiation between wolves and dogs which were grouped using only morphological traits (prior phenotypic information); and (2) to infer the presence of genetically differentiated clusters assuming that all the samples may belong to a single indistinct "population", independently of any prior classification, by means of multivariate ordination, inter-individual genetic distances and Bayesian clustering. Once distinct populations have been identified, we used Bayesian admixture analyses to assign the individuals to the populations and infer their ancestry, independently of any prior information.

Table 1. Origin, sample size and identification of the studied wolf (*Canis lupus*) and dog samples

Samples and ID	Number	Origin
Italian wolves (W)	104	Italian Apennines[1]
Dogs (D)	95	30 breeds and 8 feral dogs[2]
"Black Wolf" W334	1	Central Italian Apennines (Abruzzo)
"Black Wolf" W508	1	Northern Italian Apennines (Tuscany)
"Fifth Finger Wolf" W520	1	Siena Province (Tuscany)
Wolf-Dog Hybrids (H)	4	Captive
Captive Wolves (Wu)	5	Popoli, Pescasseroli and Caramanico Zoos

[1] For the current distribution and location of sampling areas of wolves in Italy, see: Corsi et al. 1999, and Randi et al. 2000.

[2] Detailed information on origin, population biology and ecology of the studied feral dogs are reported by Boitani et al. 1995.

Methods

Sample collection and DNA extraction

A total of 211 tissue and blood samples (Table 1) were collected from wild-living wolves distributed across the entire species' range in Italy, from dogs representing 30 different breeds and including eight feral dogs sampled in the central Italian Apennines in an area of documented sympatry with wolves (Boitani et al. 1995). We have also analysed four known wolf-dog hybrids, which were obtained in captivity, and five captive-reared wolves of unknown ancestry, which were thought to derive from matings between Italian and non-indigenous wolves (L. Boitani and P. Ciucci, personal communication). Dogs were sampled through veterinary practices, while wolf samples were obtained mainly from found-dead animals and local trapping projects. Tissue and blood samples were preserved in 100% ethanol and in a Tris/SDS buffer (Longmire et al. 1998), respectively, and stored at –20 °C. Total DNA was extracted from tissues using a guanidinium-silica protocol (Gerloff et al. 1995), and from blood using a salting-out procedure (Miller et al. 1988).

All wild-living Italian wolves showed the typical wolf coat colour pattern and phenotypical traits (Boitani 1986), excepting two unusually dark wolves ("black wolves" with identification nos W334 and W508), and a third one that showed the presence of a spur in both its hindlegs ("fifth finger wolf" labelled W520 throughout the text; P. Ciucci, personal communication). Black coats and the fifth finger are usually absent in wild-living Italian wolves and could have originate from crossbreeding with dogs or non-indigenous wolves.

The studied samples were subdivided into four groups (Table 1) according to their origins: (1) wild-living Italian wolves (labelled W throughout the text), (2) dogs (labelled D), (3) hybrids (samples H86, H102, H110 and H111), and (4) captive-reared wolves of unknown origin (samples Wu54, Wu81, Wu126, Wu314 and Wu479). All captive-reared wolves and the hybrids were excluded from analyses of genetic diversity, but their ancestry was investigated by Bayesian procedures.

Microsatellite genotyping

Eighteen microsatellites (Table 2), originally typed in the domestic dog, were selected for their polymorphism and reliable scorability in wolves and dogs (Funk et al., submitted). Three loci (CXX.213, C09.250, vWF.X) were polymorphic in European and North American wolves (Roy et al. 1994; Ellegren et al. 1996), and five loci (CPH4, CPH7, CPH9, CPH12, CPH22) showed significantly different allele frequencies in dogs and Italian wolves (Dolf et al. 2000).

PCR amplifications were carried out in 10 μl volumes (50 ng DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 $MgCl_2$, 0.1 μg BSA, 2 nmol of each dNTP, 0.25 units of *Taq* polymerase and 1–5 pmol of fluorescently labelled primers) using a Perkin Elmer 9600 thermal cycler. Cycling conditions were optimized for each primer pair. Diluted amplicons were analysed using an ABI 373A sequencer and the computer programs Genescan 2.1 and Genotyper 2.1.

Analyses of genetic variation

The distributions of allele frequencies, presence of private (group-specific) alleles, single-locus and

Table 2. A description of the microsatellite loci and summary of the allelic variation in the studied wolf and dog samples

Locus ID[1]	Chromosome[2]	Alleles[3]	Size Range[4]	H_E in Wolves[5]	H_E in Dogs[5]
CPH2[7]	S15	7	95–107	0.574	0.728
CPH3[7]	CFA6	15	161–191	0.710	0.817
CPH4[7]	S6	8	141–155	0.407	0.584
CPH5[7]	CFA15	8	112–128	0.655	0.686
CPH6[7]	CFA22	11	120–137	0.629	0.839
CPH7[7]	CFA2	9	163–183	0.244	0.789
CPH8[7]	CFA13/19	11	197–217	0.764	0.766
CPH9[7]	S3	7	144–156	0.244	0.685
CPH12[7]	CFA8	9	193–215	0.299	0.678
CPH22[8]	?	4	111–117	0.396	0.161
CXX213[9]	S10	10	149–167	0.567	0.708
C09.250[9]	CFA9	10	129–149	0.683	0.830
C20.253[9]	CFA20	7	103–115	0.200	0.402
FH2010[6]	S5	6	222–242	0.217	0.743
FH2079[6]	S5	8	263–295	0.665	0.686
FH2088[6]	S6	9	93–137	0.691	0.786
FH2096[6]	S4	6	92–112	0.664	0.579
vWF.X[10]	CFA16	6	152–188	0.558	0.662

[1] ID = locus identification as reported in the DogMap site at http://www.fhcrc.org/science/dog_genome/map/map3/lgps/syngp06.html;
[2] S = canine syntenic group; CFA canine chromosome;
[3] Total nos of alleles found in the studied wolves and dogs;
[4] Size range in base pairs, including the primers;
[5] H_E = expected heterozygosity (Nei 1987; eq. 7.39, pg. 164);
[6] Tetranucleotide loci (Francisco et al. 1996);
[7] Dinucleotide loci (Fredholm and Winteroe 1995);
[8] Dinucleotide locus (Dolf et al. 2000);
[9] Dinucleotide loci (Ostrander et al. 1993);
[10] Hexanucleotide locus (Shibuya et al. 1994).

average values of observed (H_O) and expected heterozygosity (H_E) were evaluated separately for the Italian wolves and dogs using Genepop 3.1d (Raymond and Rousset 1995; program available from http://www.cefe.cnrs-mop.fr/) and Fstat 2.9.1 (Goudet 1995; http://www.unil.ch/izea/softwares/fstat.html). Differences in heterozygosity between wolves and dogs were tested with the Wilcoxon signed rank test. We also used Genepop to test pairwise linkage equilibria at all loci over the two groups, to compute pairwise estimates of F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995). Estimators of F_{ST} and R_{ST} can be used to test the deficit of heterozygotes and the extent of divergence among populations (F_{ST} and R_{ST} significantly > 0). R_{ST} is analogous to F_{ST}, but includes differences in allele size under the stepwise mutation model. Values of F_{IS}, the coefficient of inbreeding, which estimates the deficit of heterozygotes within populations (F_{IS} significantly > 0), were computed using the method of Weir and Cockerham (1984) as implemented in Genepop. Deviations from Hardy-Weinberg equilibrium (HWE) were tested for all locus-population combinations and globally, using the Markov chain method of Guo and Thompson (1992) as implemented in Genepop. Significance levels were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice 1989). Differentiation between wolves and dogs was assessed also by Analysis of MOlecular VAriance (AMOVA; Excoffier et al. 1992) as implemented in Arlequin 2.0b2 (http://anthropologie.unige.ch/arlequin) using analogues of F_{ST} and R_{ST} (Michalakis and Excoffier 1996).

Analysis of bottleneck effects in the Italian wolves

Allelic diversity is reduced faster than heterozygosity in populations which underwent recent bottlenecks, that is within approximately $0.2N_e$ to $4N_e$ (N_e = effective population size) generations, thus bottlenecked populations are expected to show values of observed heterozygosity (H_O) larger than expected from the observed number of alleles assuming mutation drift-equilibrium (Cornuet and Luikart 1996). Genetic evidence for a bottleneck effect in the Italian wolves was evaluated using the program Bottleneck 1.1 (http://www.ensam.inra.fr/URLB), which tests whether H_O is significantly different from the expected heterozygosity at mutation-drift equilibrium (H_{EQ}). Significant differences between H_O and H_{EQ} indicate either a recent severe reduction in N_e (if $H_O > H_{EQ}$), a recent expansion in N_e or influx of rare alleles from genetically distinct immigrants (if $H_O < H_{EQ}$; Cornuet and Luikart 1996). However, extent and sign of differences between H_O and H_{EQ} depend also on the pattern of evolution of microsatellite loci (Cornuet and Luikart 1996). Bottleneck implements three tests of heterozygosity excess, each one under the Infinite-Allele Model (IAM; Maruyama and Fuerst 1985), the Two-Phase Mutation Model (TPM; Di Rienzo et al. 1994; in this study we used TPM with 95% single-step mutations and 5% multiple-step mutations), and the Stepwise-Mutation Model (SMM; Ohta and Kimura 1973).

Ordination plotting and clustering of individual genotypes

Inter-individual genetic distances, including the standard allele-sharing (DPS; Bowcock et al. 1994), and deltamu ($\Delta\mu^2$; Goldstein et al. 1995) distances, were estimated with Microsat 1.5d (http://lotka.stanford.edu/microsat/microsat.html). Distance matrices were then used to construct neighbor-joining trees (NJ; Saitou and Nei 1987) with the program Neighbor in Phylip 3.5c (http://evolution.genetics.washington.edu/phylip.html). In addition, for all allelic classes at all loci, each individual was scored as 0.0 (the allele was not observed), 0.5 (one copy of the allele was observed in heterozygotes), or 1.0 (two copies were observed in homozygotes). The matrix was then ordinated in a multidimensional space by principal coordinate analysis (PCA) using Pcagen (http://www.unil.ch/izea/people/jgoudet.html).

Bayesian clustering, admixture analysis and population assignment

Pritchard et al. (2000) described a Bayesian clustering method (implemented in the program Structure, http://www.stats.ox.ac.uk/~pritch/home.html), which uses multilocus genotypes to infer population structure and simultaneously assign individuals to populations. This model assume that there are K populations (where K may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to populations, or jointly to two or more populations if their genotypes indicate that they are admixed. This method can be used to detect the presence of cryptic population structure and to perform assignment testing. Pritchard et al.'s model assumes HWE and LE among the unlinked loci. Departures from HWE and LE lead the population to be split into subpopulations, to which individuals are assigned. In this study the posterior probabilities of K (i.e., the likelihood of K as a proportion of the sum of the likelihoods for different values of K) are estimated assuming uniform prior values on K between 1 and 5 (option MAXPOPS = 1–5). Presence of structure in the data set is revealed by the increasing likelihood of the data. The results presented in this study are based on runs of 100,000 iterations, following a burn-in period of 10,000 iterations.

Results

Analysis of genetic variation

We determined the individual genotypes at 18 microsatellite loci in 107 Italian wolves, 95 dogs and nine additional wolves of unknown origin or known hybrids (Table 1). All microsatellites were polymorphic, showing 4–15 different alleles per locus (average 8.5 ± 2.6) and values of H_E ranging from 0.161 to 0.839 (Table 2). The allele frequency distributions varied across loci and showed marked differences between wolves and dogs (Figure 1). At a threshold frequency of 5%, chosen to reduce the effects of sampling error, there were 39 private alleles, 32 in dogs and seven in the Italian wolves (Table 3).

The microsatellites used in this study map onto different dog chromosomes (DogMap Consortium 1999; Ostrander et al. 2000), except FH2010-FH2079 and CPH4-FH2088, which map onto syntenic groups S6 and S5, respectively. However, these loci should be

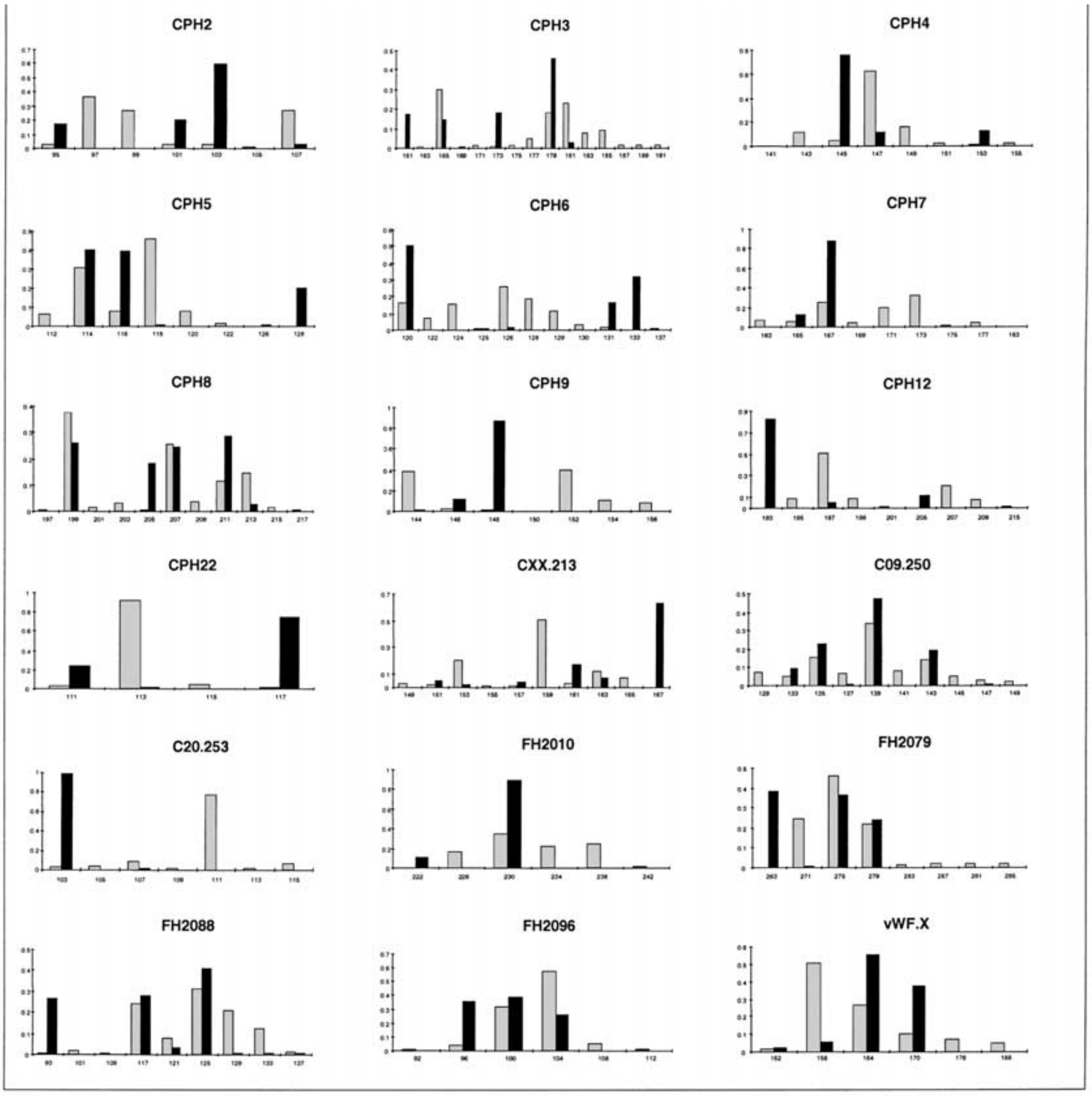

Figure 1. Histograms illustrating the frequency distributions of microsatellite lengths in wild-living Italian wolves (black bars) and domestic dogs (grey bars) for the studied microsatellite loci (see Table 2).

distant enough to allow for independent allele recombination. In fact, pairwise allelic combinations were in LE at all loci over the two groups, except in four cases in dogs (significance probability level $p < 0.05$, Bonferroni corrected for 324 comparisons).

Genetic diversity was significantly reduced in Italian wolves, which showed lower allele number and heterozygosity than dogs (Table 2). H_O was not significantly different between wolves and dogs (*Z*-value = –1.154, *p*-value = 0.248; Wilcoxon signed rank test), on average, while H_E was significantly lower in wolves (*Z*-value = –2.980, *p*-value = 0.003; Table 3). The average number of pairwise allelic differences was also lower in wolves. Dogs and wolves showed significant deficit of heterozygotes at 12 and 3 over 18 loci, respectively ($p < 0.05$; Bonferroni corrected), and, overall, loci were not in HWE. Average F_{IS} was significantly positive in both dogs ($F_{IS} = 0.241$; $p < 0.05$) and wolves ($F_{IS} = 0.101$; $p < 0.05$; Table 3). Dogs do not belong to a single breeding population, and might deviate from HWE due to inbreeding in reproductively separated breeds. Deviations from

Table 3. Summary of genetic diversity indices for the Italian wolves and dogs. Standard deviations across loci are in brackets. Departures from HWE were estimated by Fstat ($p < 0.05$)

Statistics	Wolves	Dogs
Mean No of Alleles per Locus	4.3	7.9
No of Private Alleles ($p > 0.05$)	7	32
No of Pairwise Differences	8.50 (3.94)	11.23 (5.12)
Observed Heterozygosity (H_O)	0.440 (0.210)	0.510 (0.142)
Expected Heterozygosity (H_E)	0.499 (0.216)	0.674 (0.166)
Hardy-Weinberg Equilibrium (F_{IS})	0.101	0.241

HWE due to deficit of heterozygotes may indicate inbreeding in local patches and presence of geographical population structuring in Italian wolves (Wahlund effect; Hartl and Clark 1989, pp. 282–296). However, we can not exclude that excess homozygosity was, at least in part, due to non-amplifying null alleles (Pemberton et al. 1995). Microsatellite variability was significantly partitioned between wolves and dogs ($F_{ST} = 0.33$ and $R_{ST} = 0.26$; $p < 0.001$; AMOVA). Estimates of F_{ST} and R_{ST} were similar one each other, suggesting that wolves and dogs differ similarly in distribution of allele frequency and allele size (Slatkin 1995).

Estimating the bottleneck effect in the Italian wolves

Results obtained by Bottleneck (10,000 replications) showed that: (1) the Italian wolf population was not at mutation-drift equilibrium across all loci, independently of the mutation model, using the “standardized difference test” ($p = 0.05$); (2) five and 14 loci over 18 showed heterozygosity excess and did not fit the SMM ($p = 0.010$) and the IAM ($p = 0.028$), respectively, but fit the TPM ($p = 0.342$); and (3) the one tailed Wilcoxon “sign test” for heterozygosity excess was significant ($p = 0.009$) under the IAM, but not under the TPM and SMM. Experimental evidence suggests that microsatellites evolve through complex patterns, being different at loci which have different structure of the repeated sequences or of the flanking regions (Colson and Goldstein 1999). It is not known which models might fit the evolution of the canine loci used in this study. However, the results we obtained using different tests under the assumptions of the different models, suggest that the effects of the population bottleneck in the Italian wolf population are detectable at the genetic level.

Clustering and ordination plot of individuals

The NJ tree clustering inter-individual microsatellite DPS distances (1 – ps; e.g., one minus the proportion of shared alleles; Bowcock et al. 1994) is shown in Figure 2. Wild-living Italian wolves and dogs were split into two distinct clusters. Three (H102, H110 and H111) of the four known hybrids were intermediate between wolves and dogs. However, hybrid H86 was included within the wolf cluster. Three (Wu81, Wu126 and Wu479) of the five captive-reared wolves were intermediate between wolves and dogs, but Wu54 and Wu314 were included within the wolf cluster. The three wolves with anomalous phenotypes (W334, W508 and W520) were also included within the wolf cluster. Values of bootstrap support to the main clusters were as low as 28% and 32% (Figure 2). Other genetic distances (e.g., deltamu, F_{ST} and R_{ST}) performed worst or much worst than DPS, because clusters were almost totally unresolved. Thus, NJ trees were of limited utility to identify hybrids and assign individuals of unknown origin to populations.

The results of PCA of the individual genotypes are shown in Figure 3. Individual scores were plotted onto the two principal axes (PC-I and PC-II), which cumulatively explain 32.4% of the total genetic diversity. This plotting showed a clear separation of the individual scores into two different groups on PC-I, being all dogs and Italian wolves placed on the right and left sides of the plot, respectively. Wolves and dogs were almost totally overlapping on PC-II and on all the other axes, as well. Some feral dogs (D101, D104 and D106) clustered apart in the lower right side of dogs’ distribution swarm, but they were not intermediate between wolves and dogs. The other ferals (D8, D105, D107, D108 and D109) were included within the distribution of dogs’ scores, suggesting that feral dogs have not incorporated wolf genes. All the hybrids (H86, H102, H110 and H111), plus three captive-reared wolves (Wu81, Wu126 and Wu479), were intermediate between wolves and dogs. However, the other two captive-reared wolves (Wu54 and Wu314) were included within the distribution of wolves. Two of the free-ranging Italian wolves showing anomalous phenotypic traits (“black wolf” W334 and W520) were also intermediate between wolves and dogs, while “black wolf” W508 was completely included into the wolf swarm. Therefore, excluding prior information on the origin of individual samples, PCA would have correctly identified wild-living Italian wolves and dogs as belonging to two clearly distinct groups, with all

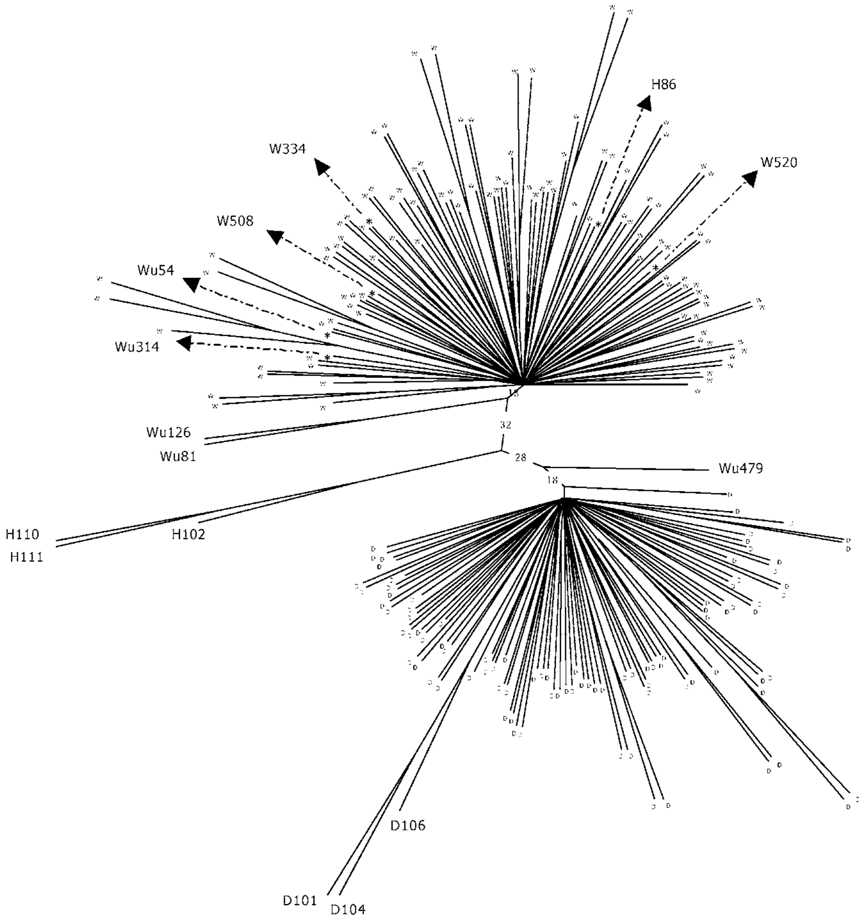

Figure 2. Unrooted network of dog and wolf samples computed using pairwise DPS distances (Bowcock et al. 1994) and the neighbor-joining clustering algorithm. Each tip represents an individual. Bootstrap support for branching pattern is indicated. The tree was constructed using Neighbor in Phylip 3.5c. The pairwise distance matrix was computed using Microsat 1.5d.

the known hybrids and some captive-reared wolves of unknown origins plotting intermediate. Two of the free-ranging Italian wolves showing anomalous phenotypes (W334 and W520) could have admixed ancestry. However, the edges of wolf's and dog's distributions were delimited visually and it is not clear if some individuals (i.e., W81 and W520) should be or not included within the Italian wolves.

Genetic admixture analysis

We used Structure with two modeling approaches. First, we assumed uninformative priors on all the K, that is we assumed that, before applying the model to the data, all samples belong to one "population" (option USEPOPINFO = 0). The individual classification based on phenotypic information was not used and all wolves and dogs were pooled into a hypothetical single "population".

The probability of the number of populations (K) for the pooled data was estimated by fixing prior values of K = 1–5 (option MAXPOPS), and comparing the Ln likelihood of the data. If the hypothetical single "population" is admixed and includes more than one subpopulation, the likelihood of the data will increase with K (Pritchard et al. 2000). Results of this analysis are reported in Table 4a. The Ln probability of the data was minimum with K = 1 (Ln = –11296.9), and maximum with K = 3 populations (Ln = –8827.6), thus suggesting that the pooled "population" of wolves, dogs and hybrids is heterogenous and may contain three genetically distinct groups.

We have, therefore, estimated the proportion of membership (q) of each predefined group into two

Table 4. (a) Probability of the number of populations (K) for the pooled wolf and dog samples (211 individuals, 18 loci); and (b) Proportion of membership of each pre-defined population in two or three inferred clusters computed using STRUCTURE

(a)

K	Ln probability of the data
1	–11296.9
2	–8878.6
3	–8827.6
4	–9066.5
5	–9127.2

(b)

Population (ID)	Two inferred clusters		Three inferred clusters		
	I	II	I	II	III
Dogs (D)	0.993	0.007	0.892	0.003	0.104
Wolves (W)	0.004	0.996	0.007	0.990	0.003
Hybrids (H)	0.571	0.429	0.489	0.403	0.108
CaptiveWolves (Wu)	0.270	0.730	0.087	0.698	0.215

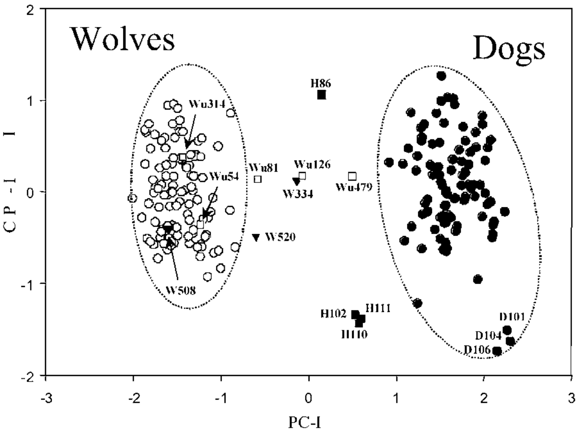

Figure 3. Scores of individual wolf and dog microsatellite genotypes plotted on the first two axes (PC-I, PC-II) of a principal coordinate analysis performed using Pcagen. H = known captive-reared hybrid wolves; Wu = captive-reared wolves of unknown origin; W334 and W508 = “black wolves”; W520 = “fifth finger wolf”.

and three clusters representing the “cryptic” genetic populations (Table 4b), that is the average proportions of genotypes in each predefinite group that are inferred to come from each population. Using two inferred clusters, cluster I grouped all dogs and cluster II all wolves, each one with proportion of membership $q = 0.99$. Thus, dogs and wolves were split into two different clusters based only on their genetic make up and independently on any prior population information. The known hybrids and captive-reared wolves of unknown origins were associated partially to both cluster I and II, in accordance with their putative admixed ancestry. The analysis with three inferred clusters confirmed a strong association of wolves to cluster II ($q_2 = 0.99$), while dogs, which were mainly associated to cluster I ($q_1 = 0.89$), showed a component associated to cluster III ($q_3 = 0.10$). Probably the strong departure from HWE in the heterogeneous dog assemblage lead Structure to identify the presence of more than one genetic cluster. The hybrids were mainly split between clusters I and II ($q > 0.40$), while the captive-reared wolves were split between clusters II ($q_2 = 0.70$) and III ($q_3 = 0.21$), suggesting that they have admixed ancestry in Italian and other populations. Using four inferred clusters dogs were still included into clusters I and III, while the captive-reared wolves were split mainly between clusters II and IV. In these study we have not included samples from non-Italian wolf populations and it was not possible to directly identify the origin of putative non-Italian wolf genotypes.

In the second modelling approach we assumed that samples should belong to one of the following four pre-defined “groups”: wild-living Italian wolves, dogs, hybrids and captive-reared wolves of unknown origin, and asked the program to assign the individuals

Table 5. Population assignment and inferred ancestry of individuals estimated using Structure. The probability (q) of each genotype to belong to one of the two clusters was computed using prior population information on the classification based on phenotypic characters. In this case Structure estimates the probability of each individual to have an ancestry either in the sampled, or first or second past generations (q-values computed with prior migration rate = 0.05)

Samples	Cluster I	Cluster II	Cluster III	Cluster IV
Dogs	≥0.99	<0.01	0.00	0.00
Wolves	<0.01	≥0.99	0.00	0.00
W334	0.00 – 0.68 – 0.32	0.00	0.00	0.00
W508	0.00	1.00	0.00	0.00
W520	0.00 – 0.00 – 0.10	0.90	0.00	0.00
H86	0.00 – 0.00 – 0.09	0.00 – 0.00 – 0.41	0.48	0.01 – 0.00 – 0.01
H102	0.00 – 0.04 – 0.06	0.00 – 0.03 – 0.09	0.79	0.00 – 0.00 – 0.00
H110	0.00 – 0.00 – 0.02	0.00 – 0.00 – 0.02	0.96	0.00 – 0.00 – 0.00
H111	0.00 – 0.00 – 0.02	0.00 – 0.00 – 0.01	0.97	0.00 – 0.00 – 0.00
Wu54	0.00 – 0.00 – 0.00	1.00 – 0.00 – 0.00	0.00 – 0.00 – 0.00	0.00
Wu81	0.00 – 0.00 – 0.00	0.99 – 0.01 – 0.00	0.00 – 0.00 – 0.00	0.00
Wu126	0.00 – 0.00 – 0.00	0.55 – 0.40 – 0.05	0.00 – 0.00 – 0.00	0.00
Wu314	0.00 – 0.00 – 0.00	1.00 – 0.00 – 0.00	0.00 – 0.00 – 0.00	0.00
Wu479	0.00 – 0.00 – 0.01	0.00 – 0.00 – 0.00	0.00 – 0.00 – 0.01	0.98

and infer the ancestry of hybrids and captive-reared wolves of unknown origins using prior population information (USEPOPINFO = 1). With two inferred clusters we force to have all wolf and dog genotypes sampled from one of the two different clusters. Structure estimates the probability of each sample to have an ancestry in the other group, either in the sampled generation, or in the first or second past generations (q-values were computed with prior intergroup "migration rate" = 0.05. In this context "immigrant" means "hybrid"). Probabilities of membership are the posterior values of q_i (i = 1, 2, 3) for each individual, that is the proportion of each individual genotype originating in one or more than one cluster. The hybrids and captive-reared wolves were probabilistically assigned to cluster I or II. Using three or four inferred clusters we forced the hybrids (predefined population 3) and the captive-reared wolves (pre-defined population 4) to have their genes sampled from different groups, and we wish to estimate their probability to have an ancestry in other groups, either in the sampled generation, or in the first or second past generations.

Results obtained using two to four clusters are strictly concordant, thus we show only results obtained using four clusters (Table 5). All dogs, including all feral dogs, were assigned to cluster I with average q_1 = 0.99, and had no significant ancestries in the other clusters. Wolves were assigned to cluster II with q_2 = 0.99, and had no significant ancestry in the other groups except for "black wolf" W334, which showed significant ancestry in dogs' first and second past generation (cluster I). The other two Italian wolves showing anomalous phenotypes ("black wolf" W508 and W520) were significantly associated to cluster II (with q_2 = 1.00 and 0.90, respectively), and had no ancestry in the other clusters. All the hybrids were assigned to cluster III and individual H86 showed significant ancestry in the second past generation of the Italian wolves (cluster II). The captive-reared wolves showed significant ancestry in the Italian wolf cluster II, except for Wu479, which was significantly associated to cluster IV. These finding suggest that Wu479 originated from (or from crossings with) non-indigenous wolves, which were not included in the studied data set.

Discussion

The historical process leading to the decline of the wolf in Europe after World War II was concomitant with an increase of free-ranging and feral dogs, which could hybridize and pollute the gene pool of wolf populations. However, the genetic data published so far failed to detect any rate of crossbreeding and introgression in the extensively studied Italian and Spanish wolves (Vilà and Wayne 1999; Randi et al. 2000), although a few cases of dog-wolf hybrids were observed in nature (Boitani 1982; Andersone et al., submitted) and detected by DNA analyses (Randi et al. 2000) in Italy and elsewhere in Europe.

The maternally inherited mtDNA can identify only wolf hybrids between female dogs and male wolves and their backcrossings. Genetic and behavioral data indicate that hybridization in *Canis* is usually asymmetric, but the direction of predominant matings and gene flow are different in different interacting species or populations (Vilà and Wayne 1999). Hybridizing wolves in eastern Canada showed coyote-like mtDNA haplotypes, while none of the studied sympatric coyotes had wolf-like mtDNA haplotypes (Lehman et al. 1991). Asymmetrical gene flow was confirmed by microsatellite data showing that, in area of sympatry in North America, allele frequences of hybridizing wolves are affected by introgression, while those of coyotes have not changed (Roy et al. 1994). Gene introgression was predominantly from coyotes into wolves either because of strictly asymmetrical matings – only female coyotes mate with male wolves –, or because first generation hybrids do not backcross into the coyote populations. Field and behavioral observations, and DNA data, suggest that dispersing female wolves predominantly mate free-ranging dogs (Boitani 1982; Randi et al. 2000), and that pregnant females are not accepted in their natal wolf packs. So their offspring may not survive in the wild and do not backcross into the wolf population (Smith et al. 1997; Vilà and Wayne 1999). A different outcome of mating asymmetry was observed in hybridizing Ethiopian wolves and domestic dogs in the Bale Mountains, where female wolves mating with male dogs are accepted into their natal packs. Their offspring can survive in the wild and are integrated into the wolf population. Consequently, first generation hybrids can backcross and transfer dog nuclear genes into the Ethiopian wolf population (Gottelli et al. 1994).

High pup mortality, which was observed in feral dogs in Italy (Boitani et al. 1995), may probably reduce survival and backcrossing rates of first generation hybrids. Phase shifting in the breeding season of first generation hybrids (Mengel 1971) may furtherly restrict their chances to backcross into the wolf population. Therefore, the probability to observe dog mtDNA haplotypes in wolves could be low also in presence of a substantial rate of hybridization. However, neither strict asymmetry in wolf's mating behavior, nor effective constraints against backcrossing have been documented in field studies, and the available genetic evidence can not rule out episodic hybridization or past introgression of domestic genes into wild-living wolf populations.

In this study we have pre-classified wolves sampled in Italy using only external morphological traits, which allowed us to identify most of the collected samples as phenotypically "pure" wolves with no apparent markings of hybridization, except for two "black wolves" (W334 and W508), and one "five fingers wolf" (W520). Microsatellite variation was significantly lower in Italian wolves than in dogs and other wolf populations studied in Europe and North America (Funk et al., submitted). Low genetic variability might be a consequence of long term isolation of wolves in the Italian Apennines south of the Alps, or of the recent demographic decline and population bottleneck. However, although the bottleneck analyses indicate that the Italian wolf population is not in mutation drift-equilibrium, the excess of observed over the expected equilibrium heterozygosity is weak and dependent on the assumed microsatellite mutation model. Moreover, the previously observed mtDNA monomorphism (Randi et al. 1995) suggested that the Italian wolves could have persisted at low effective population size (i.e., N_e = 30–50) during the last 100–120 years (Randi et al. 2000). Therefore, genetic variability could have been lost by random drift across many generations and not only during the recent population bottleneck.

Both dogs and wolves showed significant deficit of heterozygotes, positive values of F_{IS} and deviations from HWE. Similar results were reported for most of the studied North American wolf populations suggesting either non-random breeding caused by local inbreeding, or the presence of geographical subpopulation structuring (Roy et al. 1994; Forbes and Boyd 1997). Wolf packs in non-expanding populations generally consist of related individuals (Lehman et al. 1992) and, although wolves have widespread home ranges and may disperse across long distances, isolation during the bottleneck period might have lead local population fragments to diversify through genetic drift. However, the extent of geographical structuring in the Italian wolf population, as well as the genetic effects of recent population expansion and natural colonization should be more carefully assessed by analyzing a larger sample set of individuals.

Genetic diversification between wolves and dogs is quantified by values of $F_{ST} = 0.33$ and $R_{ST} = 0.26$, meaning that about 30% of the total genetic diversity is distributed between the two groups. The genetic consequences of domestication, as well as historical isolation and demographic fluctuations, have led dogs and wolves to diverge significantly in their microsatellite allele frequencies. The presence of many private alleles, some of which were at relatively high frequency in wolves or dogs, directly suggests that there has been little gene flow between the two groups, at least during the most recent generations. These data do not support the hypothesis that frequent crossbreeding with free-ranging dogs might have sustained the levels of genetic diversity in the wild-living Italian wolf population after the bottleneck (Boitani 1984).

Microsatellites were variable enough to provide unique genotypes for each of the sampled wolves and dogs. Inter-individual relationships were assessed using principal coordinate analysis of multilocus genotypes (PCA), neighbor-joining clustering of inter-individual genetic distances (NJ), and a Bayesian clustering procedure (Pritchard et al. 2000). PCA and NJ procedures split dogs and wolves into two separate clusters without using prior population information: all wolves and dogs were correctly identified by their genotypes and there were no outliers, that is none of the samples were assigned differently from the prior phenotypic classification (Figure 2 and 3). Two of the Italian wolves showing anomalous phenotypes (W334 and W520) were intermediate between wolves and dogs, while “black wolf” W508 was included into the distribution of wolves’ score in PCA. By contrast, all wolves with anomalous phenotypes were included within the wolf clade in the NJ trees. Assuming that intermediate positions mean admixed genotypes, PCA plottings, but not NJ trees, suggested that two of the free-ranging Italian wolves showing anomalous phenotypes (i.e., W334 and W520) could have admixed ancestry. However, neither the PCA nor the NJ procedures allow to estimate the uncertainty inherent to the spatial ordination and clustering.

By contrast, the Bayesian procedure jointly assigns a probability to the number of populations and to the membership of each individual to each population, allowing to extract precise quantitative information from the data set. The multilocus genotypes from individual wolves, dogs and hybrids fit the genetic model (i.e., the assumptions that genetic markers are independent, in HWE and LE) better if samples are split into at least three distinct populations (Table 4a). When samples were assigned to K = 2 inferred clusters, without using any prior population information, all dogs were assigned to cluster I and the free-ranging Italian wolves to cluster II and without significant ancestry in the other clusters. The hybrids and captive-reared wolves of unknown origins were partially assigned to more than one cluster, thus suggesting that they had admixed ancestry (Table 4b). When the ancestry of the samples was furtherly investigated using prior population information, all wolves were assigned to cluster II and showed no significant ancestry in the other groups except for “black wolf” W334, which showed significant ancestry in dogs’ first and second past generation (Table 5). The other two Italian wolves showing anomalous phenotypes were assigned to the Italian wolf cluster II and had no ancestry in the other clusters. All the hybrids were distinct and mainly associated to cluster III, except H86 which showed significant ancestry in the second past generation of the Italian wolf cluster II. These findings suggest that the captive-reared hybrids might originate from crossbreedings between dogs and wolves of non-Italian origin (cluster III), except H86, which showed mixed ancestry in Italian and putative non-Italian wolves. The captive-reared wolves showed significant ancestry in the Italian wolf cluster II, except for Wu479, which was significantly associated to cluster IV (putative non-indigenous wolf ancestors). Ancestry in non-Italian wolves should be tested by genotyping wolf samples from other Eurasian and North American wolf populations.

Additional information from mtDNA sequencing lend further support to the hypothesis that “black wolf” W334 is a hybrid. In fact W334, as well as the other Italian wolves with anomalous phenotypes (W508 and W520) showed the Italian wolf mtDNA haplotype, referred to as W14 by Randi et al. (2000). Therefore, putative hybrid W334 probably is a backcrossing of a first generation hybrid between a male dog and a female Italian wolf. All captive-reared wolves showed the Italian wolf mtDNA haplotype W14, except Wu479 that showed a mtDNA haplotype closely related to haplotypes W7 and W8, which were found in north European wolves (see: Figure 2 in Randi et al. 2000).

These findings provide, for the first time, evidence for the occurrence of rare backcrossing of wolf-dog hybrids into the Italian wolf population. Italian wolves and dogs are genetically differentiated, individuals cluster separately and can be reliably classified using either PCA, NJ or Bayesian procedures without any prior population information. However, only Bayesian procedures can reliably identify wolves of non-indigenous origins, and hybrid or introgressed individuals. The greater majority of wolves sampled from the Italian population show no phenotypic or genetic evidence of hybridization with dogs, and the introgression of domestic genes into wild-living Italian wolves is apparently very limited. However, larger sample sizes of both wolves and genes should be analysed to obtain accurate estimates of the rate of crossbreeding. We can not exclude that typing additional microsatellites would reveal admixed ancestry also in the "fifth finger" wolf W520. Bayesian clustering of multilocus genotypes is a powerful method to assign individuals to populations, but the identification of admixed individuals is more problematic (Pritchard et al. 2000). The microsatellite loci used in this study are either on separate chromosomes or widely spaced on the same chromosome. Backcrossing of first generation hybrids into the wolf population will dilute the proportion of domestic parental genotypes through the generations and linkage disequilibrium will be negligible after a few generations of backcrossing. Therefore, except for the introgressed non-recombining mtDNA, tracks of episodic hybridization in the past might have been lost, and the identification of past hybridization may require an exponentially increasing number of nuclear markers (Goodman et al. 1999).

It is noteworthy that the presence of coats darker than usual does not necessarily mean hybridization in Italian wolves. Mutations for "black" coat could maps into the Melanocortin Receptor 1gene (MC1-R; Newton et al. 2000) and might have multiple and independent origins in dogs and wolves. North American wolf populations normally show different color phases ranging from white, in the Arctic regions, to black coats, which are frequently observed in the northwest U.S. and are not though to indicate hybridization (Brewster and Fritts 1995). Although dark coats are not usually observed in west European wolves, the spreading of a recent mutation in the Italian population could have been fostered by the past demographic decline and current expansion after the bottleneck.

In conclusion, results of this study showed that Italian wolves and dogs are significantly differentiated and individual genotypes can be assigned to the two groups with high probability without using prior phenotypic information. Bayesian admixture analyses allow detection of individuals with admixed ancestry and can be used to infer the scale of introgression of dog genes into the wolf population. Application of these methods would help to implement conservation strategies for wolf populations. Despite national and international protection in most European countries, the wolf is still threatened throughout most of its range due to habitat destruction, direct persecution, accidental killing, and hybridization with dogs (Boitani 1999). The availability of diagnostic morphological, behavioral and molecular traits would help to map the regional distributions of pure wolf populations, or locate areas of introgression where populations of free-ranging dogs should be carefully controlled.

Acknowledgements

This study would not have been possible without the help of colleagues who collected and kindly provided wolf and dog samples, and in particular: L. Boitani, P. Ciucci, F. Francisci, G. Boscagli, C. Sulli, G. Dolf, A. DeMarco and many others. We thank M. Beaumont for advice in statistical analyses, L. Boitani and P. Ciucci for sharing unpublished information and comments on history, dynamics and conservation of Italian wolves. Special thanks to S. Funk for laboratory expertise and collaboration. We thank two anonymous referees for constructive comments and for helping with manuscript revision and editing. This study was supported by the Istituto Nazionale per la Fauna Selvatica (INFS) and the Consiglio Nazionale delle Ricerche (CNR).

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