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From the Apennines to the Alps: colonization genetics of the naturally expanding Italian wolf (*Canis lupus*) population

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Abstract

Wolves in Italy strongly declined in the past and were confined south of the Alps since the turn of the last century, reduced in the 1970s to approximately 100 individuals surviving in two fragmented subpopulations in the central-southern Apennines. The Italian wolves are presently expanding in the Apennines, and started to recolonize the western Alps in Italy, France and Switzerland about 16 years ago. In this study, we used a population genetic approach to elucidate some aspects of the wolf recolonization process. DNA extracted from 3068 tissue and scat samples collected in the Apennines (the source populations) and in the Alps (the colony), were genotyped at 12 microsatellite loci aiming to assess (i) the strength of the bottleneck and founder effects during the onset of colonization; (ii) the rates of gene flow between source and colony; and (iii) the minimum number of colonizers that are needed to explain the genetic variability observed in the colony. We identified a total of 435 distinct wolf genotypes, which showed that wolves in the Alps: (i) have significantly lower genetic diversity (heterozygosity, allelic richness, number of private alleles) than wolves in the Apennines; (ii) are genetically distinct using pairwise F_{ST} values, population assignment test and Bayesian clustering; (iii) are not in genetic equilibrium (significant bottleneck test). Spatial autocorrelations are significant among samples separated up to *c.* 230 km, roughly correspondent to the apparent gap in permanent wolf presence between the Alps and north Apennines. The estimated number of first-generation migrants indicates that migration has been unidirectional and male-biased, from the Apennines to the Alps, and that wolves in southern Italy did not contribute to the Alpine population. These results suggest that: (i) the Alps were colonized by a few long-range migrating wolves originating in the north Apennine subpopulation; (ii) during the colonization process there has been a moderate bottleneck; and (iii) gene flow between sources and colonies was moderate (corresponding to 1.25–2.50 wolves per generation), despite high potential for dispersal. Bottleneck simulations showed that a total of *c.* 8–16 effective founders are needed to explain the genetic diversity observed in the Alps. Levels of genetic diversity in the expanding Alpine wolf population, and the permanence of genetic structuring, will depend on the future rates of gene flow among distinct wolf subpopulation fragments.

Keywords: autocorrelation analyses, Bayesian assignment testing, *Canis lupus*, colonization genetics, spatial population structure, wolf

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Introduction

The genetic basis of natural colonization processes are poorly known (Clobert *et al*. 2001), except for aspects of the dynamics of metapopulations, which are normally fragmented in patches that have substantial local extinction and recolonization rates (Hanski 1999). The genetic consequences of demographic processes, like historical population expansions or declines, are usually assessed a posteriori, for instance using mismatch analyses (Rogers & Harpending 1992), coalescent models (Beaumont 1999), and a variety of simulation models (e.g. Currat *et al*. 2004). However, genetic data can be fruitfully used also to reveal patterns of ongoing demographic processes (Bohonak *et al*. 2001; Estoup & Clegg 2003). For instance, the genetics of colonization may be dominated by bottlenecks if populations are founded by small numbers of colonists. Random drift might lead to further losses of genetic variability if colonies do not quickly expand and remain isolate from their source populations. Founder effect, isolation and protracted low rates of gene flow might then reduce genetic diversity and the potential for adaptation, concomitantly increasing inbreeding and the probability of extinction of colonizing populations (Hedrick & Kalinowski 2000). Genetic data allow estimating the minimum number of colonizers that are needed to explain the genetic variability observed in the colonies (Bellinger *et al*. 2003). Revealing the genetic patterns of ongoing population expansion and recolonization might have important roles also in practical conservation biology (Mcdonald & Johnson 2001), particularly if parental structure, inbreeding levels and sex-biased dispersal is assessed within and among population patches (Clobert *et al*. 2001).

Wolves have been progressively eradicated throughout Western Europe and in the Alps in the 18th and 19th centuries (Breitenmoser 1998), surviving in fragmented populations in Iberia and Italy (Blanco *et al*. 1992; Boitani 2003). Wolves in Italy were confined south of the Po River since the turn of the last century, continuing to decline until the 1970s, when *c*. 100 individuals ranged in two fragmented areas in central-southern Apennines (Zimen & Boitani 1975). Due to a more effective legal protection and substantial changes in the ecology of mountain areas (e.g. decrease of human density and increase of wild ungulates), this declining demographic trend quickly reversed in the 1980s, when wolves started to expand in Italy and in other European countries (Breitenmoser 1998; Wabakken *et al*. 2001; Boitani 2003). In Italy wolves crossed the northern Apennines and recolonized the southwestern Alps, where genetic identification confirmed their presence since 1992 in France and 1996 in Switzerland (Valière *et al*. 2003), and reappeared again in the central Italian Alps in 2000. Nowadays the Italian wolf population is guessed to number more than 600 individuals (Boitani 2003). Wolf reappearance in the Alps alleviates conservation risks and contributes to restore structured ecological communities, raising nevertheless social conflicts, which are fuelled by the occurrence of livestock predations (Duchamp *et al*. 2004). Thus, wolf expansion and colonization need to be carefully monitored. Studies in Europe and North America revealed that colonization may be sustained by long-distance dispersers (Valière *et al*. 2003), and that colonizing wolves may not lose significant fractions of their original genetic variability (Forbes & Boyd 1996). Sporadic, but recurrent migration increased the heterozygosity and sustained population growth in a recently founded small wolf population in Scandinavia (Vilà *et al*. 2003). Results from noninvasive genetic monitoring programmes revealed that the Alps are being recolonized by naturally expanding Apennine wolves (Lucchini *et al*. 2002; Valière *et al*. 2003). Wolves and free-ranging dogs hybridize sporadically in Italy, and expanding Apennine wolf populations do not show substantial dog gene introgression (Randi & Lucchini 2002; Verardi *et al*. 2006).

In this study we used 12 autosomal microsatellite loci to genotype DNA samples extracted from wolf tissues and scats collected in the Apennines and in the Alps from 1982 to 2004, aiming (i) to assess the extent of genetic differentiation between the Apennine source and Alpine new wolf populations; (ii) to estimate the rates of gene flow that are sustaining the ongoing recolonization of the Alps; and (iii) to infer by simulation the minimum numbers of colonizers that could explain the observed population genetic parameters. We used island models, based on F_{ST} values (Weir & Cockerham 1984) estimated between predefined population groups, continuous population models, based on isolation-by-distance and autocorrelations (Smouse & Peakall 1999), and Bayesian clustering models (Falush *et al*. 2003). Describing the genetic structure of newly founded populations and the patterns of diversification between sources and colonies would help understanding the prevalent dispersal patterns, and the composition and dynamics of wolf packs in newly colonized areas.

Materials and methods

Sample collection and DNA analyses of microsatellite markers

DNA was extracted from tissues, collected mainly from the source wolf populations in the central and southern Apennines, and scat samples collected noninvasively from the populations which recently expanded in the northern Apennines and recolonized the Alps (Table 1). Tissue samples from the source populations were collected by the Italian Wildlife Institute (INFS) and the Department of Animal and Human Biology of the University of Rome 'La Sapienza' over the last 20 years from wild-living wolves accidentally or illegally killed in Italy. All these animals had the typical Italian wolf coat colour pattern and did not

Table 1 Origin, number and sex (M, male; F, female; U, undetermined; Lucchini *et al*. 2002) of wolf (*Canis lupus*) samples genotyped in this study. Population groups include samples collected from the Alps (Piemonte in Italy; Alpes Maritimes, Alpes de Haute Provence and Hautes Alpes in France; Graubünden and Valais in Switzerland); northern Apennines (NAps; Liguria, Emilia Romagna, northern Tuscany, and northern Marche); central Apennines (CAps; southern Tuscany, southern Marche, Umbria, Abruzzo, Lazio and Molise); southern Apennines (SAps; Campania, Calabria, Basilicata and Puglia)

Population	Number and kind of samples	Number of distinct genotypes	Collectors (years)
Alps	885 scats	130 (68M; 62F)	Progetto Lupo Piemonte (1999–2004)
	11 tissues	11 (9M; 2F)	Life Nature in France (1999–2004)
			KORA in Switzerland (1998–2003)
NAps	2000 scats	122 (69M; 53F)	Life Nature Emilia-Romagna (2000–2004)
	25 tissues	25 (13M; 12F)	Regione Emilia-Romagna (2000–2004)
			INFS (2002–2004)
CAps	126 tissues	126 (62M; 56F; 8U)	INFS & University of Rome (1982–2002)
SAps	21 tissues	21 (9M; 10F; 2U)	INFS & University of Rome (1982–2002)
Total	3068 samples	435 (230M; 195F; 10U)	

show detectable morphological and genetic signal of hybridization with dogs (Randi *et al*. 2000; Randi & Lucchini 2002). Tissue samples from France and Switzerland were also obtained from dead animals, by field correspondents of the French Wolf-Network (coordinated by the Office National de la Chasse) and of the Swiss Wolf Project (coordinated research projects for the conservation and management of carnivores in Switzerland (KORA)). Moreover, we analysed 2885 scats, which were noninvasively collected in the northern Apennines (Emilia-Romagna, Life Nature project) and in the Alps (Italy, France and Switzerland; progetto Lupo Piemonte, coordinated by the Regione Piemonte, Life Nature in France and KORA in Switzerland; Table 1), mainly in 2000–2004. We have subdivided these samples in four groups (hereafter referred to as four 'populations'; Table 1): wolves collected in the northern, central or southern Apennines, and in the Alps (Fig. 1). Although there is no obvious geographical break in wolf distribution, or barrier to dispersal, this subdivision is aimed to group separately those samples that were collected from the two areas in central and southern Apennines where the species survived during the bottleneck in the 1970s, and that were collected from the two areas of recent expansion in the northern Apennines and in the Alps. Tissue and scat samples were stored at –20 °C in 95% ethanol. DNA was extracted using a guanidinium-silica protocol (Gerloff *et al*. 1995) or with the QIAGEN Stool kit (QIAGEN), and genotyped by polymerase chain reaction (PCR) at 12 microsatellites: six dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; Fredholm & Wintero 1995; C09.250; Ostrander *et al*. 1993), and six tetranucleotides (FH2004, FH2079, FH2088, FH2096, FH2132 and FH2137; Francisco *et al*. 1996). Amplifications were carried out in 10-μL reactions, using, respectively, 1 or 5 μL DNA solutions from tissue or scat extractions, plus 1 μg of BSA. PCR conditions were optimized for each primer pair and for tissue or scat samples (laboratory details are available upon request).

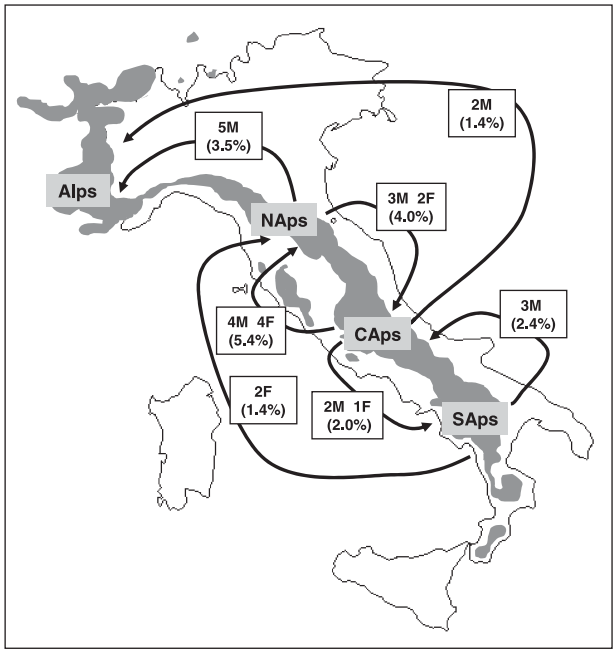

Fig. 1 Approximate wolf (*Canis lupus*) distribution range in Italy and in the Alps (in grey). Dotted areas indicate regions of temporary wolf presence in north France and Switzerland. The regions of origin of the sampled wolves in the Alps, north (NAps), central (CAps) and southern Apennines (SAps) are indicated. The arrows indicate the directions (with the number, sex and percent) of first generation migrant wolves that were detected using the Rannala & Mountain's (1997) method as implemented in GENECLASS (Piry *et al*. 2004).

Excremental DNAs were extracted and amplified in a separate room only dedicated to low-DNA-content samples, genotyped through a multiple-tube protocol (Taberlet *et al*. 1996; Lucchini *et al*. 2002), followed by reliability analysis (Miller *et al*. 2002). Negative (no DNA in PCR) and positive

(samples with known genotypes) controls were always used. PCR products were analysed using an ABI 3100 automatic sequencer and the ABI software GENOTYPER version 3.7.

Genetic variability analysis

We used the software GENALEX version 6 (Peakall & Smouse 2005) to estimate allele frequency by locus and population, observed (H_O) and expected unbiased (H_E) heterozygosities, mean number of alleles per locus (N_A), number of private alleles (N_P) per population (i.e. the number of alleles unique to a single population in the data set). GENALEX was also used to perform the following procedures:

1 AMOVA (analysis of molecular variance; Excoffier *et al*. 1992; Michalakis & Excoffier 1996) was used to assess the extent of global and pairwise population differentiation based on ϕ_{pt}, an analogue of F_{ST}, which estimates the proportion of variance among populations, relative to the total variance.
2 The frequency-based population assignment test developed by Paetkau *et al*. (1995) was used to detect the most probable population of origin of each individual by comparing the likelihood of each multilocus genotype in a set of predetermined populations. Additional Bayesian assignment testing (Rannala & Mountain 1997), and detection of first-generation migrants (Paetkau *et al*. 2004) were performed using the software GENECLASS version 2 (Piry *et al*. 2004). Moreover, we used the Bayesian program STRUCTURE version 2.1 (Falush *et al*. 2003) to infer cryptic population structure and simultaneously assign individuals to populations, independent of any prior nongenetic information.
3 Multivariate spatial autocorrelation analyses and a Mantel test (Smouse & Paekall 1999) were used to detect spatial structuring through correlations between pairwise geographical and genetic distance matrices. Allelic richness, which corrects the observed number of alleles for differences in sample sizes, was computed with FSTAT version 2.9.3.2 (Goudet 1995). The inbreeding estimator Wright's F_{IS} (Weir & Cockerham 1984), and tests for departures from Hardy–Weinberg equilibrium, were computed using the software GENETIX 4.03 (Belkhir *et al*. 2001).

Founder effects and bottleneck detection

We hypothesize that the ongoing process of wolf colonization involved a population bottleneck, which can be designed as follows: a small number of colonizers moved *c*. 20–15 years ago (1990) from the source population in the Apennines, through the narrow corridor along the ridge of the northwestern Apennines (Liguria), and entered in the southern Italian (Liguria and Piemonte) and French Alps (Alps Maritimes), finally arriving in Switzerland in 1996. To assess the strength of bottleneck we used Cornuet & Luikart's (1996) test as implemented in BOTTLENECK version 1.2.02 (Piry *et al*. 1999), which is based on the expectation that in a recently bottlenecked population the observed heterozygosity is higher than the heterozygosity expected from the observed number of alleles under the assumption of a population at mutation–drift equilibrium. This expectation holds only for loci evolving under the infinite allele model (IAM), not necessarily for markers following a strict stepwise mutation model (SMM). Under an intermediate two-phase model (TPM), a heterozygosity excess is expected after a bottleneck. Luikart & Cornuet (1997) recommended the TPM for microsatellites, with a predominance of one-step mutations and a small percentage (P = 5–10%) of multistep changes. In this study we genotyped 15–40 individuals per population with 12 loci. We tested the bottleneck effect under the TPM with P = 10%, and using the sign, the one-tailed Wilcoxon, and the L-shaped distribution tests.

The effects of a population bottleneck on genetic variability were simulated using BOTTLESIM (Kuo & Janzen 2003), aiming to estimate how many colonizers are needed to explain the variability observed in the Alpine wolf population. We specified the following parameters: diploid multilocus, variable population size, completely overlapping generations, random mating system, 8 years of expected organism longevity, age of reproduction of 1 year, sex ratio 1/1. We simulated (i) the effects of bottlenecks of variable size (from a minimum of 2 up to 20 founders) in newly founded populations, which remain stable up to 8 years (corresponding to the first three generations after the beginning of wolf colonization in the Alps); and (ii) the effects of bottlenecks of variable size (from a minimum of 4 up to 14 founders) in newly founded populations, which grew for 14 years (corresponding to the first five generations after the beginning of wolf colonization in the Alps) at a rate per year variable between 7% and 21% (Ciucci & Boitani 1991). A precise estimate of the Apennine wolf population is not available, so we assumed a source population size of 300 individuals, which genetic variability was described by the genotypes that were sampled in north and central Apennines. Values of average N_A, H_E and fixation index (F), which were obtained after 100 iterations in each simulation, were plotted and compared with corresponding values observed in the Alpine wolf population. Moreover, we compared the simulated and real F_{ST} values computed between the Alpine and the north Apennine wolf populations.

Results

Genetic variability in wolf populations

We have determined a total of 435 distinct wolf genotypes using 12 unlinked microsatellites (Table 1). All loci were

Table 2 Genetic diversity in wolves genotyped at 12 unlinked microsatellite loci. Populations: Alps, Italian, French and Swiss Alps; NAps, northern Apennines; CAps, central Apennines; SAps, southern Apennines (n, number of distinct genotypes). H_O, observed heterozygosity; H_{Eunb}, unbiased expected heterozygosity; N_{Amean}, mean number of alleles per locus (direct count); N_{AR126}, allelic richness estimated by rarefaction (using FSTAT; Goudet 1995) based on a minimum sample size n = 126 (number of genotypes in central Apennines); N_P, number of private alleles. Standard errors in parentheses. Departures from Hardy–Weinberg equilibrium were assessed for each population from average multilocus F_{IS} values (the average individual inbreeding coefficient within each population); P, probability to obtain F_{IS} values lower (for negative F_{IS}), or higher (for positive F_{IS}) than observed after 1000 random permutations of alleles in each population determined using GENETIX (**P < 0.01; *P < 0.05)

Populations (n)	H_O	H_{Eunb}	N_{Amean}	N_{AR126}	N_P	F_{IS}	P
Alps (141)	0.57 (0.03)	0.56 (0.03)	3.50 (0.38)	3.56	0	−0.028	0.096
NAps (147)	0.60 (0.03)	0.62 (0.03)	5.42 (0.75)	5.36	7	0.037**	0.010
CAps (126)	0.62 (0.04)	0.64 (0.04)	5.25 (0.76)	5.21	5	0.035*	0.017
SAps (21)	0.59 (0.02)	0.59 (0.04)	4.08 (0.51)	—	0	0.018	0.310

polymorphic in all populations, showing intermediate values of heterozygosity (H_O = 0.57–0.62; H_E = 0.56–0.64), and average number of alleles per locus ranging from 5.42 (in north Apennines) to 3.50 (in the Alps; Table 2). Values of H_E were significantly different among the four populations (P = 0.005; Friedman test), due to significantly lower values in the Alps vs. northern Apennines (P = 0.023; Wilcoxon signed-rank test) and vs. central Apennines (P = 0.020). Wolves sampled in the Alps showed approximately two alleles per locus less than in the Apennines, on average. Allele richness, estimated by rarefaction for a sample size n = 126 (corresponding to the number of genotypes sampled in central Apennines) was significantly lower in the Alps vs. both northern and central Apennines (P = 0.002; Friedman test). Alpine wolves again showed approximately two alleles per locus less than Apennine wolves. Wolves in the Alps and southern Apennines had no private alleles, while there were, respectively, seven and five private alleles at low frequency (< 0.02) in north and central Apennine wolves. Wolves in the southern Apennines were globally in Hardy-Weinberg equilibrium (multilocus test), wolves in northern and central Apennines showed a slight deficit of heterozygotes (significantly positive F_{IS} values), while wolves in the Alps showed a slight excess of heterozygotes (nonsignificant negative F_{IS} value). Results of single-locus Hardy-Weinberg equilibrium tests showed that heterozygote deficit was contributed only by loci FH2137 and FH2088, which were both significant in the Alps, while only locus FH2137 was significant in the central Apennines (data not shown). Heterozygote deficit at FH2137 was detected both in the Alps (genotypes obtained from scats) and in central Apennines (genotypes obtained from tissues) thus ruling out significant allelic dropout biases in noninvasive samples. Only locus CPH2 showed a significant heterozygote excess, which determined the negative F_{IS} value in the Alps. Low genetic diversity and slight excess of observed vs. expected heterozygotes in wolves in the Alps might signal the consequences of a population bottleneck during the colonization process.

Table 3 Pairwise values of F_{ST} (below the diagonal) and derived estimates of N_m (above the diagonal) among four wolf populations

Population	Alps	NAps	CAps	SAps
Alps		2.44	2.74	1.60
NAps	0.093		15.01	3.21
CAps	0.084	0.016		5.51
SAps	0.135	0.072	0.043	

Genetic divergence and gene flow assuming an island model and genetic equilibrium

A significant average multilocus F_{ST} = 0.09 (P = 0.01; computed from AMOVA) indicates that genetic diversity was significantly partitioned among the four wolf groups. All pairwise F_{ST} values (Table 3) were significant (P < 0.001). The largest F_{ST} values were observed between the Alps and the Apennines, which consequently showed the smallest effective F_{ST}–based migration rates. Assuming genetic equilibrium, wolves in the Alps should exchange $N_m = [1/(F_{ST} - 1)]/4 = 2.44$ effective individuals per generation with the northern Apennines source populations (Table 3). Gene flow was lowest between the Alps and southern Apennines (N_m = 1.6), and highest between north and central Apennine wolves (N_m = 15.1). Despite potential high dispersal and gene flow, wolves sampled in distinct areas in Italy are significantly differentiated, suggesting that the ongoing population expansion process is sustained by limited gene flow, and that formerly isolated populations have not completely admixed yet.

Detecting geographical structure using continuous population models

The population assignment test implemented in GENECLASS (Rannala & Mountain's method, with the 'leave-one-out' option, that is omitting the sample that is being assigned to the populations) showed that 342/435 (78.6%) wolves

Table 4 Results of the population assignment test performed using the Rannala & Mountain's (1997) method, with the 'leave-one-out' option as implemented in GENECLASS (Piry *et al*. 2004). The table indicates the number (and percentage) of wolf genotypes which were sampled in a region, and that were assigned to the sampling or to any other region

	Assigned to			
Sampled in	Alps	NAps	CAps	SAps
Alps: 141	131 (93%)	6 (4%)	4 (3%)	0
NAps: 147	4 (3%)	111 (75%)	26 (18%)	6 (4%)
CAps: 126	2 (2%)	28 (22%)	84 (67%)	9 (9%)
SAps: 21	0	0	5 (24%)	16 (76%)

Table 5 Average proportion of membership (Q_I; computed using STRUCTURE; Falush *et al*. 2003) of individual wolf genotypes grouped in four predefined populations, and assigned to two ($K = 2$) or four ($K = 4$) inferred clusters

	$K = 2$		$K = 4$			
Given population	Q_I	Q_{II}	Q_I	Q_{II}	Q_{III}	Q_{IV}
Alps	0.897	0.103	0.194	0.033	0.049	0.724
NAps	0.156	0.844	0.297	0.451	0.222	0.030
CAps	0.124	0.876	0.122	0.298	0.525	0.055
SAps	0.085	0.915	0.037	0.113	0.814	0.036

were correctly assigned to their sampled populations (Table 4). Among the 93 wolves assigned to other populations there were 10 wolves sampled in the Alps that were assigned to the north and central Apennines, and 6 wolves sampled in north and central Apennines that were assigned to the Alps. No wolves were exchanged between Alps and south Apennines. The assignment test showed that population subdivision in north and central Apennines had weak genetic support because misassignment proportions are high (*c*. 20%). About 24% of wolves sampled in southern Apennines were assigned to the central Apennine populations, but none to the north Apennines. Also wolves in southern Apennines are genetically distinct. The numbers (and percentages) of inferred first-generation migrants (males and females), as identified by GENECLASS using Paetkau *et al*.'s (2004) procedure, are reported in Fig. 1. These data show that the colonization of the Alps was sustained mainly by wolves dispersing from the north Apennine population (five first-generation migrants detected), and to a minor degree from the central Apennine population (two first-generation migrants). Wolves from the southern Apennines did not contribute to the recolonization of the Alps. Migration was male-biased because all inferred first-generation migrants to the Alps were males. Results of the assignment tests indicate that wolves in the Alps are differentiated, perhaps due to bottleneck effect and reduced gene flow during the ongoing recolonization process. Migration was a unidirectional process, with wolves moving mainly from the Apennines to the Alps.

Population subdivision was confirmed by STRUCTURE. The posterior probability of the data [LnP(D)] was estimated from four replicated runs for a number of subpopulations $K = 1$–10, each one of 10^4 burn-ins followed by 10^5 iterations, using the admixture model and correlated allele-frequencies (*F*-model; Falush *et al*. 2003). The optimal K value was identified using a procedure described by Garnier *et al*. (2004). Results showed an increase of likelihood values up to $K = 4$. The first population split, obtained assuming $K = 2$, separated the Alpine vs. the Apennine wolves, which were, respectively, assigned to cluster I with probability $Q_I = 0.90$, or to cluster II with probability $Q_{II} = 0.84$–0.92 (Table 5). Minor proportions of wolf genotypes sampled in the Alps or in the Apennines were assigned to the other clusters with $Q_I = 0.10$–0.16. Subsequent population splitting, obtained with $K = 3$ and $K = 4$ (Table 5) separated wolves from the southern Apennines, and confirmed the genetic distinction of the Alpine wolves.

Patterns of geographical population structure were quantitatively assessed by autocorrelation analyses, which showed a significant ($P < 0.01$) negative regression between individual genetic distances and individual geographical distances, meaning that geographically close wolves showed genetic distances (and thus relatedness) higher than by chance. Significantly positive correlation coefficients r values were obtained among samples that are separated up to *c*. 230 km (Fig. 2a). This is the value of the first x-intercept that provides an estimate of the nonrandom genetic structure present in the data. Multiple autocorrelation analyses for increasing class sizes showed that r decreased with increasing the size of distance classes. The distance class at which r is no longer significant, would approximate the extent of detectable spatial genetic structure. An autocorrelation analysis performed using the four population groups (Alps, NAps, CAps and SAps) as distance classes further support the conclusion that within group genetic similarity is higher at the extremes than at the centre of the wolf distribution (Fig. 2b). A Mantel test showed a significant global correlation between individual genetic distance and geographical distance matrices ($R = 0.219$; $P = 0.001$).

Bottleneck effect and simulations of the colonization patterns

Results of Cornuet & Luikart's (1996) bottleneck test, computed under the TPM with $P = 10\%$, showed that only the Alpine wolf sample had significant heterozygote excess, using either the sign test ($P < 0.05$) and the Wilcoxon test

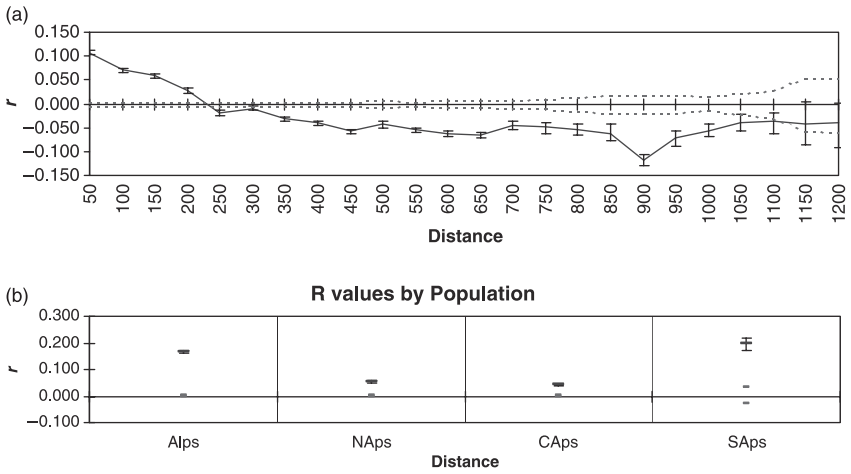

Fig. 2 Correlograms showing the genetic correlation *r* as a function of distance (kilometres) among wolf populations, computed using GENALEX (Peakall & Smouse 2005); (a) autocorrelations for distance class size of 100 km; (b) autocorrelations performed using four population groups (Alps, north, central and southern Apennines) as distance classes. The 95% confidence intervals about the null hypothesis of a random spatial distribution of genotypes (broken lines), and 95% confidence error bars about *r* values were calculated for each distance class by bootstrap.

($P < 0.01$), while in all populations the allele frequency distribution was L-shaped, as expected in equilibrium populations. Thus, founder effect and limited gene flow during the colonization process produced a detectable bottleneck signal in the Alpine wolf population. We used BOTTLESIM to estimate the minimum number of founders that could have colonized the Alps, conditional to the average number of alleles per locus, expected heterozygosity and F_{ST} values between Alps and north plus central Apennines. In these simulations, we used the 273 distinct genotypes sampled in north and central Apennines, which, according to the results of population structure analyses, can be considered as the source population of the Alpine wolves. A first set of simulations was designed to test a bottleneck effect assuming that Alpine wolves did not expand. We assumed that the population originated from 2 to 20 founders and did not expand for the following eight years. Results showed that the average number alleles per locus $N_A = 3.50$ (standard error = 0.03) observed in the Alpine population needs at least six effective founders (Fig. 3a). However, alleles will be lost by drift if the new population remain stable for up to 8 years (corresponding to *c*. three wolf generations), if the number of founder was less than 14–16 (not shown). The other two parameters, H_E (Fig. 3b), and *F* (Fig. 3c), support these conclusions, suggesting that the observed genetic diversity would need a colonization by *c*. 12–16 effective wolves. If we assume that the colonizing wolf population started to grow immediately at an average annual rate of *c*. 7%, the genetic diversity observed after 14 years (corresponding to 4–5 wolf generations) would need a colonization by at least eight founders. Also the generation of an observed $F_{ST} = 0.08–0.09$ between the new wolf population in the Alps and the source populations in north and central Apennines would need a minimum number of about eight founders. We have tested different rates of annual increase (7%, 14% and 21%) but we did not observe significant difference in N_A, H_E, *F* and F_{ST} values (data not shown).

Discussion

Origin of the Alpine wolf population

Mitochondrial and microsatellite DNA analyses of found-dead wolves and scat samples collected in Italy, France and Switzerland concordantly indicate that wolves colonizing the Alps originated from the natural expansion of Italian Apennine wolf populations (Lucchini *et al*. 2002; Valière *et al*. 2003). Wolves collected in the Alps share the diagnostic mtDNA control-region haplotype (named W14 by Randi *et al*. 2000) that is unique to the Italian wolf population. Assignment testing of multilocus microsatellite genotypes corroborates this conclusion, showing that wolves collected in the Alps closely match the genotypes of wolves collected in the Apennines (Lucchini *et al*. 2002; results in this study). No wolf × dog hybrid genotype has been sampled in the Alps, so far. Overall, these findings confirm that the ongoing recolonization of the Alps has not been determined, or at least strongly sustained by unofficial releases of captive-reproduced wolves of non-Italian origin, or by widespread hybridization with dogs. Such notwithstanding, wolves in the Alps are genetically distinguishable from their source population living in the Apennines, either at the individual and at the population level. Bayesian assignment (GENECLASS) and clustering analyses (STRUCTURE) allow to correctly assign 90–93% of the individual Alpine wolves to their sampled Alpine population. Population substructuring is evidenced by population assignment procedures (Table 4). Spatial population substructuring has been quantitatively detected by autocorrelation analyses

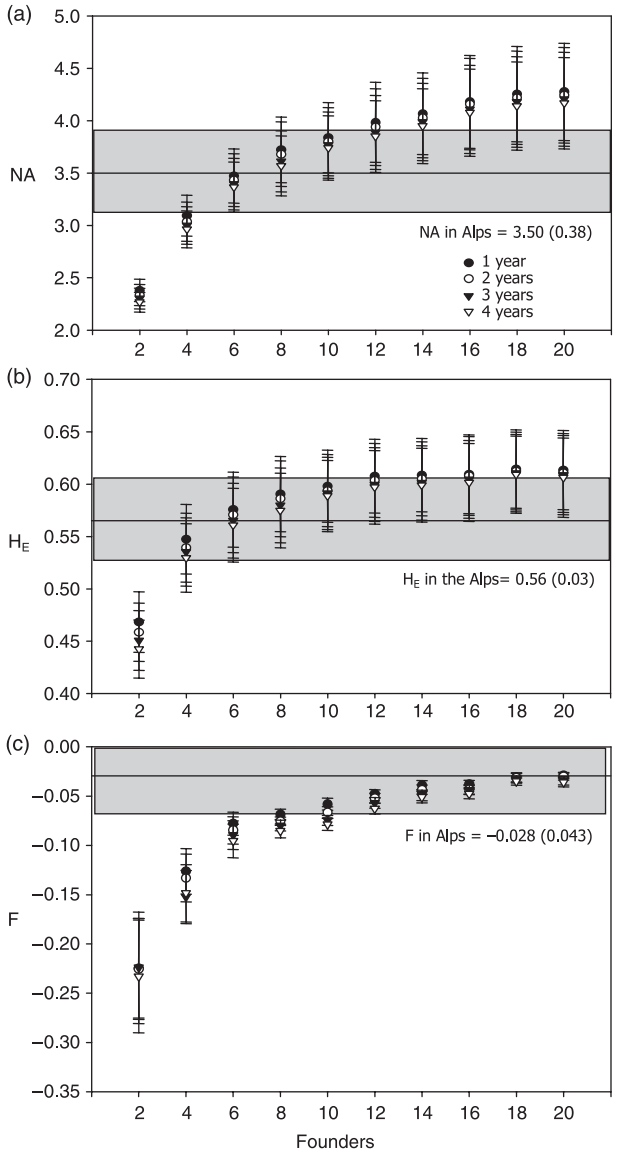

Fig. 3 Bottleneck simulation obtained using BOTTLESIM (Kuo & Janzen 2003). Effects of bottlenecks of variable size (from a minimum of 2 up to 20 founders) on the average number of alleles per locus (N_A; a), the expected heterozygosity (H_E; b) and the fixation index (F; c), in newly founded populations that remain stable for 1–4 years. Dark bars indicate the standard errors of the observed values in the Alpine wolves.

(Fig. 2). These findings suggest that population substructuring in wolves in Italy has been determined by founder and bottleneck effects at the onset of the recolonization process of the Alps.

Founder effect during the recolonization of Alps

The genotypes identified in this study showed that wolves sampled in the Alps have significantly lower genetic diversity (in terms of heterozygosity, allelic richness and number of private alleles) than wolves from the Apennines. Genetic diversity has been lost during the bottleneck that occurred at the onset of the recolonization process, as indicated by a bottleneck test (BOTTLENECK), which is significant only in the Alpine wolf population. However, the observed loss of genetic diversity is not high: 66% of the allelic richness and 90% of the expected heterozygosity of the source population is still maintained in wolves in the Alps after about 16 years, corresponding to about 4–5 wolf generations (Aspi *et al*. 2006) from the onset of the recolonization. Hence, the founder effect was not very strong and/or the rate of gene flow during these first generations of recolonization was not very low. Accordingly, N_m estimates computed from F_{ST} values (assuming genetic equilibrium, which is not the case), or from direct count of first generation migrants obtained through a Bayesian assignment procedure (GENECLASS), which does not assume any genetic equilibrium, revealed that *c*. 1.25–2.50 genetically effective migrants per generation moved from the Apennines to the Alps. Interestingly, wolves in the Alps did not show any private allele, indicating that all allelic diversity originated from an incomplete sampling (due to bottleneck) of the Apennine source populations. Wolves in north Apennines showed seven private alleles that apparently were not spread to the Alps by migrant wolves. Each of the alleles found in the Alps was detected also in the Apennines, further excluding any genetic contribution of non-Italian wolves to the Alpine colony. The moderate strength of bottleneck is supported by simulations (BOTTLESIM), which indicates that 8–16 effective founders are needed to explain the genetic diversity currently observed in the Alps. In conclusion, these findings reveal that the Alpine wolf population was not founded by a single breeding pair, but from several genetically unrelated colonizers.

Historical decline and bottleneck effect in other wolf populations

Population genetics of wolves that recently colonized the central Rocky Mountains and southern Sweden has been studied by Forbes & Boyd (1996) and Flagstad *et al*. (2003). Colonizing wolves in North America showed high genetic variation and long-range dispersal distances, suggesting that the new packs were founded by multiple unrelated individuals, without any detectable bottleneck at founding. Recolonization and the origin of a stable breeding population following long-range dispersal were clearly revealed by mtDNA and nuclear markers typed in wolves in Sweden. In this case, recolonization was initiated by a single wolf pair, which migrated and reproduced in southern Sweden, more than 900 km far from the closest source populations in Finland and Russia. Recolonization in Sweden has been sustained by subsequent immigration of a single individual wolf, which introduced additional genetic variation

determining a positive 'rescue effect' in a severely inbred population (Vilà *et al*. 2003). Thus, long-distance dispersal and multiple founding events seem to characterize the genetics of the wolf colonization events that have been described so far.

Aspi *et al*. (2006) documented high levels of genetic diversity in the now quickly expanding wolf population of Finland, which from the 1920s to the 1970s was reduced just to a few individuals as consequence of overhunting. Expected heterozygosity values estimated in different temporal Finnish wolf samples (H_E = 0.66–0.69) were higher than heterozygosity in the Italian population (H_E = 0.56–0.64). The data set analysed by Aspi *et al*. (2006) was obtained by typing 10 microsatellites, six of which (CPH2, CPH4, CPH8, CPH12, FH2088 and FH2096) are in common with the panel used in this study. The Italian and Finnish data set are thus closely comparable, further suggesting that wolves in Italy might have been isolated for long periods of time south of the Alps (Lucchini *et al*. 2004).

The causes of population subdivisions in the Italian and in other wolf populations

Despite high potential for dispersal and gene flow, wolves sampled in distinct areas in Italy are significantly differentiated, suggesting that the ongoing population expansion is sustained by limited gene flow, and that formerly isolated populations have not completely admixed yet. At their bottleneck before the 1970s only about 100 wolves were estimated to leave in Italy, fragmented into two geographically semi-isolated subpopulations in mountain areas of the central and southern Apennine (Zimen & Boitani 1975). A large area of unsuitable wolf habitat, which separate the southern and central Apennines sections of the population (Corsi *et al*. 1999), appears to limit, though not prevent, wolf movements across the two subpopulations. Results of genetic analyses in the present study indicate that the ongoing population expansion process did not led wolves in central and southern Apennines to admix completely. Population assignment analyses show that wolves in the southern Apennines are genetically distinct from both wolves in the Alps and in central Apennines (Table 5). Founder effect and limited gene flow during the recolonization of the Alps led to the observed genetic distinction between the northern Apennine source and the Alpine colony populations. Spatial autocorrelations show significant positive *r* values among samples that are separated up to *c*. 230 km (Fig. 2). This distance is roughly correspondent to the apparent gap in permanent wolf presence between the Alps and north Apennine**s**. Ecological continuity between the northern Apennines and the Alps is maintained by the narrow corridor of the Ligurian Apennines: low human density and widespread distribution of potential large ungulate prey provide suitable habitat for wolf dispersal, but the landscape is fragmented by several large highways and other infrastructures perpendicular to the corridor. Wolf dispersal along this corridor has been shown by radiotracking (Ciucci & Boitani, personal communication), but the source population in the northern Apennines is restricted to a narrow range and the number of dispersing animals is likely to be very small. The future permanence of population substructuring will be dependent of the rates of gene flow, which will ultimately be controlled by landscape features, constraining the viability of the Apennine corridor. As long as the gene flow will be maintained at the current estimated level (1.25–2.50 individual per generation), the future of the Alpine wolf population appears to be safe from the most severe consequences of a bottleneck. However, continuous monitoring of its genetic structure and of the gene flow are necessary to ensure its conservation. Significant genetic differentiation between neighbouring wolf populations separated just by a river has been described in Canada by Carmichael *et al*. (2001), which suggested that differential hunting strategies may have prevented admixture of geographically close wolf packs specialized to hunt on resident or migratory ungulates. Isolation by distance on a continental scale has been described in wolf populations in North America (Geffen *et al*. 2004), revealing that habitat factors and topographical barriers might limit historical rates of gene flow. Pilot *et al*. (2006) showed that genetic differentiation among local wolf populations in eastern Europe was correlated with climate, habitat types, and wolf diet composition. Thus, despite the potential to disperse for up to 1000 of kilometres (Mech & Boitani 2003), wolf populations can remain isolated for periods of time due to a variety of behavioural (dispersal in familiar landscapes; intraspecific competition; prey specialization), ecological or geographical factors. Results in the present study demonstrate also that isolation by distance can be generated by bottleneck at founding and by limited gene flow during wolf population expansion and colonization of new areas.

Conclusions

This study documents some genetic aspects of a wolf recolonization process, showing that: (i) wolf recolonization of the Alps apparently involved a significant founder effect; (ii) genetic data shows instances of a moderate bottleneck; (iii) genetic differentiation is at least temporarily maintained by reduced gene flow between source and colony populations; (iv) gene flow is prevalently unidirectional and sex biased, that is male wolves move preferentially from the northern Apennines to the Alps; (v) colonization and population substructuring are both generated and maintained by recurrent but infrequent dispersal events through narrow corridors or partially suitable habitats; (vi) in consequence, local inbreeding

hotspots are generated, meaning that genotypes in close spatial proximity are more similar than genotypes with greater spatial separation. Population dynamics associated with colonizing events may have important influences on the evolutionary trajectory of newly established populations. Non-equilibrium conditions are created when a new population is founded. The main variables influencing how the new population is differentiated are the number of founder individuals, duration of small population size and demographic expansion of colonies, and patterns of gene flow (migration) after the colonization (a single main founding event or recurrent migration?). Further studies should include the extension of the monitoring program of the Alpine colonization, in order to assess if protracted low rates of gene flow will reduce the genetic variability of wolf populations in the Alps, and inflate genetic divergence between sources and colonies.

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