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ORIGINAL PAPER

Fine-scale genetic structure suggests low levels of short-range gene flow in a wolf population of the Italian Apennines

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Abstract We investigated local gene flow in a high-density wolf (*Canis lupus*) population of the Italian Apennines, where no effective barrier to wolf dispersal was present. From 1998 to 2004 we examined wolf carcasses and non-invasively collected samples, focusing on three mountain districts, separated by two valleys, where wolf packs showed high spatial stability. Using nine autosomal microsatellites we successfully genotyped 177 samples, achieving the identification of 74 wolves. Genetic relatedness steeply decreased with increasing distance between sampling areas, thus suggesting that short-distance inter-pack migration is infrequent in this population. In addition, no individual from a central pack under intensive monitoring was sampled in the range of the surrounding packs over a 4-year period. The limited short-distance gene flow resulted in a cryptic genetic structure, which was revealed by Bayesian analysis. A different genetic cluster was found in each of the three mountain areas, and a small proportion of first-generation immigrants was detected. Overall, the present study suggests that local genetic differentiation in Italian wolves might arise from high spatial stability of packs and can be favoured by a combination of long-range dispersal, the attitude to mate between unrelated individuals and a high young mortality rate.

Keywords *Canis lupus* · Autosomal microsatellites · Cryptic genetic structure · Dispersal · Non-invasive genotyping

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Introduction

Gene flow is mediated by dispersing individuals whose movements are affected by population density and habitat connectivity. In turn, dispersal patterns influence abundance, age and sex structure, and genetic relatedness within a population. Short dispersal distances are more frequent, but natal long-range dispersal is very important in recolonization processes and in shaping the overall genetic structure of a population (Ibrahim et al. 1996).

Many studies have shown that wolf packs are generally composed of a breeding pair and their offspring, but more complex pack structures can result from a variety of social mechanisms (Mech and Boitani 2003). For instance, the occurrence of non-breeding adults which are unrelated to the breeding pair seems to be not a rare event in wolf packs, especially in exploited populations (Grewal et al. 2004; Jedrzejewski et al. 2005). Mortality is indeed one of the most important determinants of dynamism in the social structure of a population, and its effects are stronger if it involves breeders (Brainerd et al. 2008).

The nature of dispersal in wolf populations is more controversial, particularly on account of the distance travelled by wolves and to a possible bias between sexes.

Dispersing wolves are able to cover more than 1,000 km (Wabakken et al. 2007), but the proportion of long-range dispersers becoming successful breeders seems to vary significantly among areas (Kojola et al. 2006). Whether the attitude toward long-range dispersal is selected in wolves is debatable, as isolated populations with frequent short-range interpack dispersal are able to retain their levels of heterozygosity and inbreeding over time (Vonholdt et al. 2008).

There is no agreement as to whether dispersal patterns in wolves are sex biased. Field data on radio-collared wolves show slight or no difference in dispersal patterns between sexes (Mech 1987; Gese and Mech 1991; Kojola et al. 2006), whereas male-biased dispersal is suggested by genetic investigations carried out in North America and Europe (Lehman et al. 1992; Seddon et al. 2005; Fabbri et al. 2007; Vonholdt et al. 2008).

In a saturated population, the formation of a new pack is expected to be quite rare, while the recruitment of alien individuals by existing packs appears to be more frequent (Vonholdt et al. 2008). The latter is the case when a non-breeding member is adopted or a breeder is replaced following its death or usurpation. Moreover, high mortality rates would be likely to promote turnover of individuals within packs and increase the frequency of splitting and merging processes.

The isolated Italian wolf population is recovering after a bottleneck that occurred in the 1960s–1970s (Cagnolaro et al. 1974). It has stably re-colonized the Western Alps and has reached a continuous distribution along the Apennines, where wolf packs can locally reach high densities. Even if the species is fully protected by the Italian law, high levels of induced mortality, mainly due to poaching and traffic accidents, are reported (Lovari et al. 2007). No study has been focused so far on the knowledge of social dynamics acting at the local level in the Italian wolf population and of the way these mechanisms are affecting its recovery.

In this study, we investigated the fine-scale genetic structure of a wolf population in the Northern Apennines. Wolves have rapidly recovered in the area during the last 20 years and by the early 2000s it appeared to have been settled by a number of close packs (Apollonio et al. 2004; Capitani et al. 2006). In the area, there is no significant barrier to wolf dispersal and human induced mortality is suspected to be high (Capitani 2005). According to Vonholdt et al. (2008), given these conditions, short-range migration of individuals might be favoured and high genetic homogeneity is expected to arise in the population. In the present study, we specifically tested the following predictions: (1) individual movements to close pack territories are frequent, as a result of short-range dispersal, pack splitting and merging/adoption processes, (2) genetic relatedness is high between neighbouring packs and slowly decreases with increasing distance, and (3) no substantial genetic structure can be found in the population.

Methods

Study area and wolf population

The study was conducted in a mountain area of the central Apennines, in the province of Arezzo, Italy. Major mountains, rarely exceeding 1,500 m a.s.l., are protected as part of a national park (Foreste Casentinesi N.P.) and five natural reserves, and are mostly covered by forests. Villages are concentrated in the lowlands and in the two main valleys, where the Arno and Tevere rivers flow (Fig. 1). The area harbours an abundant and diverse community of wild ungulates, comprising wild boar *Sus scrofa*, roe deer *Capreolus capreolus*, red deer *Cervus elaphus*, fallow deer *Dama dama*, and mouflon *Ovis orientalis musimon*. Here wolves are thought to have never disappeared (Cagnolaro et al. 1974) and since the early 1980s they have rapidly spread, from the national park (Apollonio et al. 2004), where they have profited from a rich community of wild ungulates (Mattioli et al. 1995, 2004). By 1998, several packs had occupied all major mountains as well as several surrounding hilly areas.

The local wolf population was monitored continuously from January 1998 to December 2004 by direct observation, wolf-howling and snow-tracking (see Apollonio et al. 2004; Capitani 2005). Nine to 12 packs (average 10.3±1.2) were annually counted in the province of Arezzo. Pack size was between two and eight, averaging 4.0±0.6 (mean ± sd) in winter. An overall winter density of 2.3 wolves per 100 km^2 and an average distance of 11.1±1.8 km between the rendezvous sites of neighbouring packs were estimated in the area (Capitani et al. 2006). High pack productivity and high fidelity to summer rendezvous sites were ascertained during the study period (Capitani 2005).

Sample collection and DNA extraction

Sample collection focused on three mountain districts (A to C), where wolf presence was stable, and on a few peripheral areas, settled by wolves during the study (Fig. 1). Four hundred ninety-eight samples were processed, 30 of which corresponded to wolf carcasses recovered in the study area. Age was determined for 23 dead individuals, most of which resulted to be pups or yearlings (17/23=74%).

Almost all samples were collected in the period 1998–2004. Nine carcasses had been sampled before the study period (1991–1997). Tissue (n=18), plucked hairs (n=12), shed hairs (n=157), scats (n=272), and blood spots (n=35) were used as sources of genomic DNA. Fresh (<1 week) faecal samples were collected in wolf areas, mostly along snow tracks, and stored in polypropylene tubes filled with 96% ethanol. Shed hairs were stored dry in plastic or paper

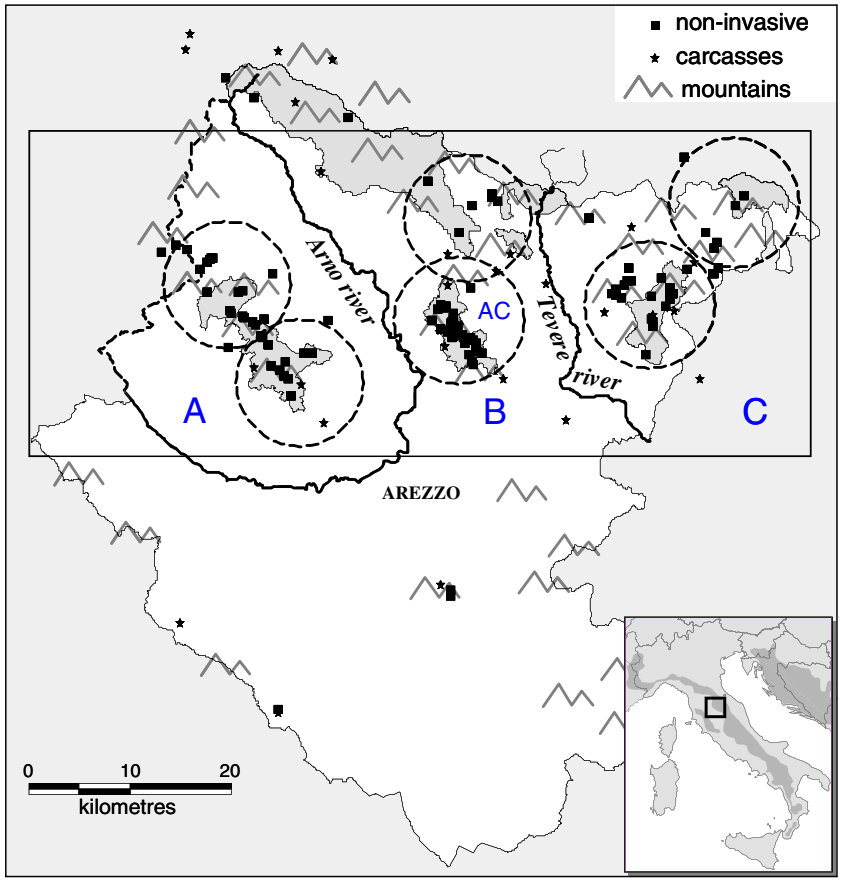

Fig. 1 Simplified map showing the distribution of genotyped samples in the province of Arezzo (Italy). Protected areas are in *dark grey*. A selected area (*rectangle*) was partitioned by the presence of two rivers into sub-areas *A*, *B*, and *C*. *Hatched circles* represent sampling units based on approximate pack locations (6-km buffer around rendezvous sites recorded in summer 2002). The intensively monitored pack of Alpe di Catenaia (*AC*) is in central position. The geographic location of the study area in relation to wolf range (*shaded*) in Italy is shown in the box (*bottom right*)

envelopes. Blood spots were collected along wolf tracks in snow and treated as previously reported (Scandura 2005). Whenever possible, all samples were kept cold immediately after their collection and then stored at −18°C until analysis.

The Chelex method (Walsh et al. 1991) was employed to isolate DNA from one to ten follicles per hair sample. QIAamp Tissue and Stool kits (Qiagen) were used to extract DNA from tissues and blood, and from excremental samples, respectively. Suppliers' protocols were followed in all cases except for melted blood/snow mixtures (see Scandura 2005). Precautions were taken to minimize contamination risk of low-copy DNA samples. DNA isolation and amplification were performed in separate rooms. Polymerase chain reactions (PCRs) were prepared in a dedicated laminar flow hood and the workspace was sterilized before each preparation. Blank extractions were conducted to monitor possible contaminations. Negative (no DNA) and positive controls were included in each PCR tray.

Microsatellite genotyping and genotype reliability

Nine canine autosomal microsatellites (dinucleotides cxx.109, cxx.123, cxx.204, cxx.250 from Ostrander et al. 1993, cxx.377 from Ostrander et al. 1995, and tetranucleotides FH2004, FH2054, FH2137, and FH2175 from Francisco et al. 1996) were selected for wolf genotyping. One primer in each pair was end-labelled with fluorescent dyes (Applied Biosystems). Amplification protocols are reported in Scandura et al. (2006). Amplicons were visualized in 2% agarose gel and then run in an ABI PRISM 3100-Avant automatic sequencer. Allele lengths were determined using the GENEMAPPER v. 3.2 software (Applied Biosystems).

Genotyping errors are likely to occur in all datasets, especially in those including poor DNA samples (Pompanon et al. 2005). DNA from different sources (i.e. excrements, hairs, blood, etc.) are subject to different error rates (Beja-Pereira et al. 2009). In a previous study (Scandura et al. 2006), we demonstrated that the probability a wolf genotype is liable to contain errors can be predicted from the quality of single-locus amplifications. In so doing we attributed a quality score (Q-score) to each genotype in a standardized framework and calculated the corresponding expected error rate for that genotype (see Scandura et al. 2006).

Here, with the aim to prevent genotyping errors in the final dataset, a multi-step protocol was adopted, considering that both high- and low-quality DNA samples were used.

1. All samples were genotyped once. A Q-score was attributed to each single-locus genotype on the basis of

the amplification profile, and the expected error rate (EER) was estimated using the regression function in Scandura et al. (2006). Then, the expected multi-locus reliability was computed as 1-Π EER_i (where i indicates the locus). Only genotypes showing an expected reliability higher than 0.5 were retained, while low-quality genotypes were discarded.

2. A multi-locus genotype was considered reliable after the first set of PCRs if at least one of the following conditions was met: (1) it was obtained from a high-quality sample with an expected reliability >0.9, (2) it was shared by two or more samples collected in the same place and the corresponding match probability for siblings (Woods et al. 1999) was <0.01.
3. All other genotypes were replicated until each allele was confirmed by at least two (heterozygotes) or three (homozygotes) independent repetitions.
4. Any possible ambiguity associated to pairs with only one or two mismatching alleles was resolved by further amplifications at the mismatching loci.
5. Finally, the software MICRO-CHECKER (Van Oosterhout et al. 2004) was used to detect possible biases in the data, which might be due to trivial typographic errors, scoring errors or null alleles.

Species identification and molecular sexing

As two other canids are sympatric with wolves in the study area, the domestic dog and the red fox (*Vulpes vulpes*), species attribution should be diagnosed for all genotypes derived from non-invasive samples. Due to genetic peculiarities of the Italian wolf, remarkable differences exist in the allelic range and frequencies at the typed microsatellites (Iacolina et al. 2010). A Bayesian clustering method was used to perform an assignment test in STRUCTURE 2.1 (Pritchard et al. 2000), using reference wolf, dog and red fox genotypes (100,000 iterations as burn-in period and 100,000 iterations for data collection, assuming three populations, i.e. K=3, and the admixture model). For each uncertain genotype the probability (q) to be assigned to each inferred cluster was estimated. Genotypes were attributed to the wolf population if the probability of assignment to the corresponding cluster was $q_{[\mathrm{wolf}]} \geq 0.95$.

Gender of non-invasively sampled individuals and non-sexed wolf carcasses was determined by a canid-specific amplification of the DBX/DBY region, following Seddon (2005). Conditions of the multiplexed PCR matched those for microsatellites, except for primer concentration (0.15 μM for DBX, 0.10 μM for DBY) and amplification profile (40 cycles of touchdown with annealing at 60–50°C). PCR products were then electrophoresed on a 2% agarose gel containing ethidium bromide for visual detection. Each set of reactions included a male control (Y-positive), a female control (Y-negative), and a blank (no template).

Statistical analysis

Genetic diversity in the population was evaluated using all available wolf genotypes and calculating the mean number of alleles per locus (k), expected unbiased (Nei 1978) and observed heterozygosity. GENETIX v. 4.02 (Belkhir et al. 2001) was used to calculate per-locus and overall F_{IS} (Weir and Cockerham 1984) in the population and to test the significance of F_{IS} values over 10,000 permutations of alleles throughout the dataset. Departures from Hardy–Weinberg equilibrium and linkage equilibrium were tested using GENEPOP 4 (Raymond and Rousset 1995) and sequential Bonferroni correction so as to account for multiple tests (Sokal and Rohlf 1995).

We used pairwise genetic relatedness to study how the signal of co-ancestry changes as distance increases. As estimator of genetic relatedness, Queller and Goodnight's (1989) pairwise relatedness R was calculated from genotype data in GENALEX v. 6 (Peakall and Smouse 2005). This analysis was restricted to individuals sampled during the period 2000–2003 in the territories of six packs inhabiting three mountain areas separated by two rivers (A, B, and C in Fig. 1 and schematic view in Fig. 2). The presence of two adjacent packs was ascertained in each area during this period. Rendezvous sites used by packs were localized on the basis of summer howling records (field techniques and methods are described in Capitani et al. 2006). We then defined sampling units (SU) as circular areas centred in the detected rendezvous site of a pack and having a radius of 6 km (Fig. 1). This distance approximates half the mean distance between adjacent packs in the study area (Capitani et al. 2006). A genotyped individual was assigned to the SU, where the geographical centre of all its sampling locations in a year fell. Once affiliation to SUs was established, pairwise relatedness values were computed for dyads belonging to the following distance classes (Fig. 2): (1) within the same unit (WSU), (2) between units in the same area (BSU), (3) between units in neighbouring areas (BNA), *iv*) between units in distant areas (BDA). In order to avoid the misleading effect of changes in pack composition from year to year, only pairs of wolves that had been sampled in the study area during the same year were considered. A total of 21 year-packs were taken into account in the calculations. Mean relatedness values obtained for different distance classes were tested for significant differences by randomization test (Manly 1991), using a Monte Carlo simulation in POPTOOLS (Hood 2006).

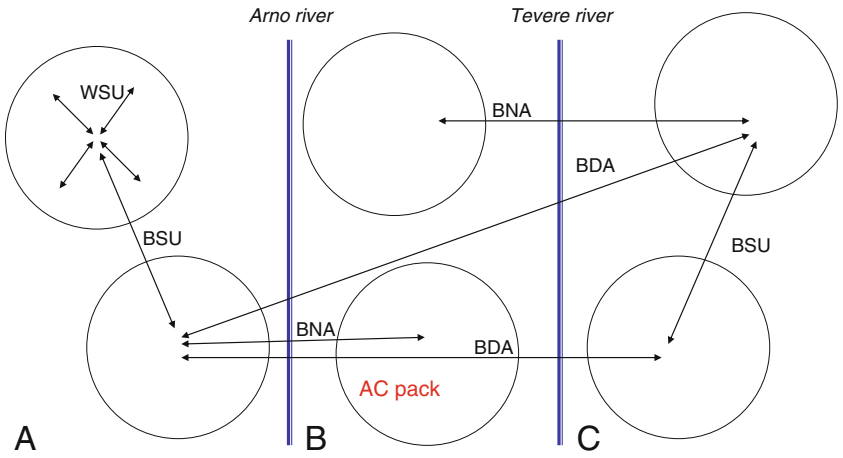

Fig. 2 Scheme showing the four distance classes that were used to classify dyads of sampled wolves by genetic relatedness: within social units (*WSU*), between units in the same area (*BSU*), between units in neighbouring areas (*BNA*), and between distant areas (*BDA*). Each circle represents a sampling unit (*SU*). The three areas correspond to sub-areas *A*, *B*, and *C* appearing in the rectangle in Fig. 1. They are separated by two rivers (Arno and Tevere)

Secondly, we studied the possibility that individuals had dispersed from a central and stable pack and entered the surrounding packs in the province. The Alpe di Catenaia (AC) pack (Fig. 1) was intensively monitored during the study period and the location of its rendezvous site was found to be nearly the same in the years 2000–2003 (see Capitani et al. 2006). Kinship was inferred from allelic compatibility and LOD scores using CERVUS v. 3 (Marshall et al. 1998; Kalinowski et al. 2007), among all individuals that were found in the corresponding SU during the same period. Pack composition derived from genetic data was further corroborated by the association among genotypes found along the same winter trail in snow. Once the breeding pair in the AC pack was determined, we sought genotypes, in the whole study area, which could be offspring of that pair.

Finally, the occurrence of dispersal in the study area was studied by a Bayesian clustering analysis in the software STRUCTURE v. 2.1 (Pritchard et al. 2000). This program estimates the log likelihood, Pr ($X|K$), i.e., the probability of the data associated with a certain number of subpopulations (K). Values of K comprised between two and ten were tested, considering that ten was the average number of packs yearly detected in the province. Convergence was obtained by 100,000 replications following a burn-in period of 100,000 iterations (admixture model, alpha=1, correlated allele frequencies). Five runs were performed for each value of K to test the consistency of results. The optimal value of K was inferred from results of simulations, following Garner et al. (2004). On account of the genetic structure that emerged in the population, the contribution of each inferred cluster to the genetic background of individuals was used to identify first-generation immigrants. In so doing, as we were considering an open population the same way as a closed one, a strict definition was adopted. Immigrant wolves were those showing a high (>90% or >75%) membership to a genetic cluster which was the most frequent in a different area (i.e. the sampling area was other than the area of origin). This analysis aimed to identify possible short-range dispersal events, and therefore it was restricted to the three areas (A, B, and C in Fig. 1) and to the time period (2000–2003) for which most data were available. Due to the geographic scale of our sampling, this approach can be considered effective in detecting cases of immigration into one area, but the origin of a given individual could not be assessed with certainty (because of the number of unsampled potential sources that lay outside the study area).

Results

A total of 177 multi-locus genotypes were obtained: 30 (17%) derived from wolf carcasses and 147 (83%) from non-invasively collected samples. MICRO-CHECKER did not detect any bias in our dataset, that could be attributed to errors in allele scoring, allelic dropout or null alleles. Scored genotypes were attributed to 86 different individuals ($P_{sib}=6.7\times10^{-5}$–2.1×10^{-2}). Among them, the Bayesian cluster analysis revealed 4 foxes and 8 dogs, each with a confidence of assignment >95%. In contrast, all other uncertain genotypes were attributed to wolves, even though two of them showed signs of a possible event of hybridization with dogs in their ancestry ($P_{[wolf]}<95\%$). Molecular gender determination allowed us to establish that 36 individuals were males and 31 were females, while the sex could not be assessed for seven individuals. A total of 55 alleles were observed at nine loci (three to nine per locus), while average H_o and H_e across loci were 0.647 and 0.660, respectively. The population departed from HWE (Fisher's method, $p=0.0021$), but this was not due to inbreeding ($F_{IS}=0.0199$, $p>0.10$). Six out of 36 pairs of loci showed a significant association at the genotypic level (linkage disequilibrium), albeit none of them was located on the same chromosome. Presumably, departure from both HWE and LE resulted from population structuring, as suggested by the simultaneous occurrence of different rare alleles in different sampling areas.

On average, each genotype deriving from non-invasive sampling was obtained from the analysis of 3.02 samples (Table 1). However, 50% individuals were sampled just once (Fig. 3a). Approximately 80% of all resampling events occurred in the same SU where a wolf was sampled for the first time; no difference was observed between sexes (Fig. 3b). With respect to the geographical partition in Fig. 1, only one wolf (male W58) was sampled in more than one area, namely in C during 2002 and in B during 2003 and 2004 (Tab. 1). Three individuals, all males, were sampled for more than 3 years (W11, W18, and W19). Male W11 was non-invasively monitored for six consecu-

Table 1 Wolves sampled between 1998 and 2004, identified by individual ID, sex and number of sampling events (N)

Genotype	Sex	N	1998	1999	2000	2001	2002	2003	2004	Area	Info
W18	M	6		❶		❷❷		❶		A	
W23	M	1			❶x					A	injured by vehicle collision and recovered by CFS
W29	F	1			❶					A	
W20	-	1		❶						A	
W32	M	2				❶		❶		A	
W33	F	8				❶❶	❶❷❷	❶		A	
W34	M	3				❷❶				A	
W35	F	2				❶❶				A	
W40	M	1					❶x			A	cub (3-4 months), driven
W42	M	1					❶			A	
W43	-	1					❶			A	
W44	M	5					❶❸	❶		A	
W10	M	1	❶x							B	2-3 years old, killed by snare
W11	M	10	❶	❷	❷	❷❶❶		❶		B	
W21	F	3		❷	❶					B	
W27	M	1			❶					B	
W26	F	1			❶x					B	> 1 year old, shot
W37	F	1				❶				B	
W38	F	5				❷	❷	❶		B	
W39	F	1				❶				B	
W45	M	15					❺❾❶			B	
W46	M	7					❹❷❶			B	
W50	F	3					❷❶			B	
W48	M	1					❶x			B	yearling (1-2 years), shot
W60	F	1						❶		B	
W66	-	1						❶		B	
W74	F	1							❶x	B	cub (8 months), shot
W12	F	1	❶x							B	3-4 years old, black fur, shot
W25	M	1			❶x					B	> 1 year old, shot
W53	M	1					❶			B	
W54	F	2					❶❶			B	
W55	M	3					❷❶			B	
W65	F	1						❶x		B	cub (6-7 months), driven
W71	-	1						❶		B	
W73	M	1						❶x		B	
W31	M	7			❶		❺❶			C	
W14	M	1	❶							C	
W15	-	1	❶							C	
W19	M	7		❶	❷	❶	❶	❶❶		C	
W47	F	1					❶			C	
W28	F	3			❶	❷				C	
W30	F	2			❶	❶x				C	cub (< 1 year), shot
W36	F	2				❶❶x				C	cub (< 1 year), poisoned
W41	M	1					❶x			C	cub (5-6 months), driven
W49	F	1					❶x			C	cub (7-8 months), unknown cause of death
W51	F	5					❷❶	❷		C	
W52	M	1					❶x			C	cub (7-8 months), unknown cause of death
W56	F	1					❶			C	
W58	M	8					❶	❶❶	❺	C, B	
W57	M	3					❶	❷		C	
W70	M	1						❶		C	
W68	M	1						❶		C	
W69	F	1						❶		C	
W72	M	1							❶x	C	driven?
W13	F	1	❶x							OUT	yearling (1-2 years), shot
W16	M	1		❶						OUT	
W17	F	1		❶						OUT	
W22	M	1			❶x					OUT	cub (< 1 year), driven?
W24	-	1			❶					OUT	
W67	M	1						❶x		OUT	cub (< 1 year), unknown cause of death
W61	M	1						❶x		OUT	cub (5-6 months), shot
W59	M	1						❶x		OUT	> 1 year old, driven?
W62	F	1						❶		OUT	
W63	M	1						❶x		OUT	> 5 years old, shot
W64	F	2						❶❶		OUT	

Shadows define the minimum period of presence of the wolf in the area. Numbers in filled circles indicate the number of sampling events in a single month. Death of monitored individuals is indicated by an "error mark" and corresponds to the finding of its carcass. For these individuals, additional information concerning cause of death and age is reported. Geographic areas (A, B, or C) where wolves were sampled refer to those reported in Fig. 1 ("OUT" indicates that the wolf was sampled out of the rectangle)

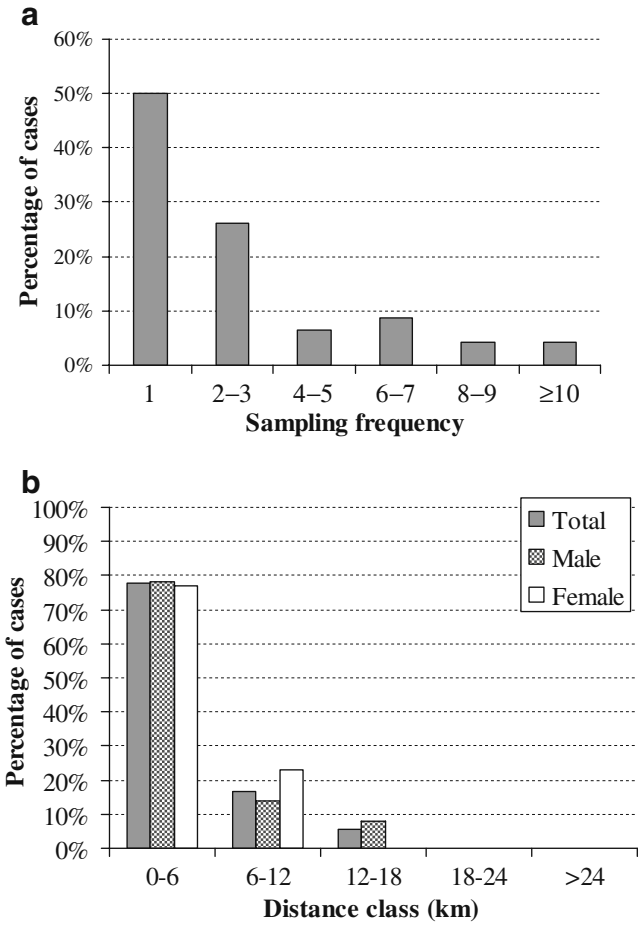

Fig. 3 Resampling of individual wolves in the study area. **a** Percentage distribution of sampling frequency. **b** Percentage distribution of classes of resampling distance (total and divided by sex)

tive years (the entire study period) in the same area where female W38 was monitored for three consecutive years, from 2001 to 2003.

As expected, relatedness in the population decreased with increasing distance (Fig. 4). In intra-pack comparisons (WSU, n=63), relatedness averaged 0.310±0.042 (mean ± standard error), but it decreased to 0.133±0.034 between packs of the same area (BSU, n=73). Pairwise relatedness dropped to average values of 0.011±0.018 when individuals sampled in neighbouring areas were considered (BNA, n=217), and it was negative (−0.124±0.023, BDA, n=90) in comparisons between distant areas. The observed differences in mean relatedness between classes were all significant (randomization test, P<0.01 for all comparisons). Hence, the signal of relatedness was lost just a few kilometres away from a SU.

According to CERVUS analysis and pair-wise relatedness values, W11 and W38 were compatible to be the parental pair of the AC pack during this period. Six individuals in our sample were likely to have been sired by this breeding pair. Five of them had been sampled between 2000 and 2003 within the AC sampling unit, while the sixth individual (W48) was a yearling that was found dead in December 2002, at 13 km distance from the AC rendezvous site. This wolf had been shot dead, but its carcass was found on the verge of a provincial road. Arguably, a post-mortem translocation had occurred. In addition, it was found in a site that was out of any wolf territory.

The Bayesian analysis allowed the evaluation of gene flow among areas and the consequent genetic differentiation within the population. The partition into three clusters (K=3) received the highest support from simulations ($\Delta\ln P(D)_{k=3}$=83.7; $\Delta\ln P(D)_{k=2}$=49.5; $\Delta\ln P(D)_{k=4}$=55.4). The contribution of the three inferred groups to the genetic composition of each sampled wolf is shown in Fig. 5. On average, individuals in area A showed 88% membership to cluster I, those in area B showed 75% membership to cluster III, and those in area C showed 64% membership to cluster II. A total of 29 wolves out of 47 (62%) were sampled in the area which was most likely (>90% probability) to be their area of origin. In area C all these 'resident' wolves were from the same SU (thus possible members of the same pack), but this was not true for areas A and B. On the other hand, three to six wolves turned out to be first-generation immigrants, applying respectively the 90% and the 75% criteria (Fig. 5). According to the previous result, no wolf from area B appeared to have dispersed to neighbouring areas. On the contrary, all immigrants but one (W44, 75% criterion) were likely to have dispersed from area A.

Discussion

In presence of very limited data on spatial behaviour by wolves in Italy, the present study provides the first genetic

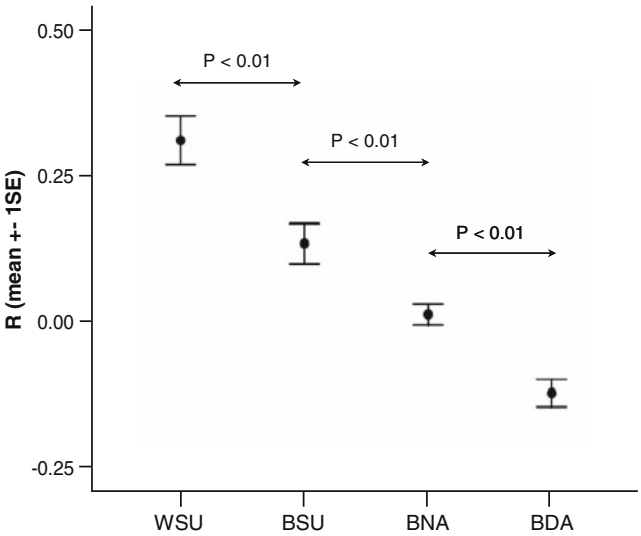

Fig. 4 Variation in the pairwise genetic relatedness between wolves belonging to different distance classes (see Fig. 2). Significance of the difference between mean values was obtained by randomization tests

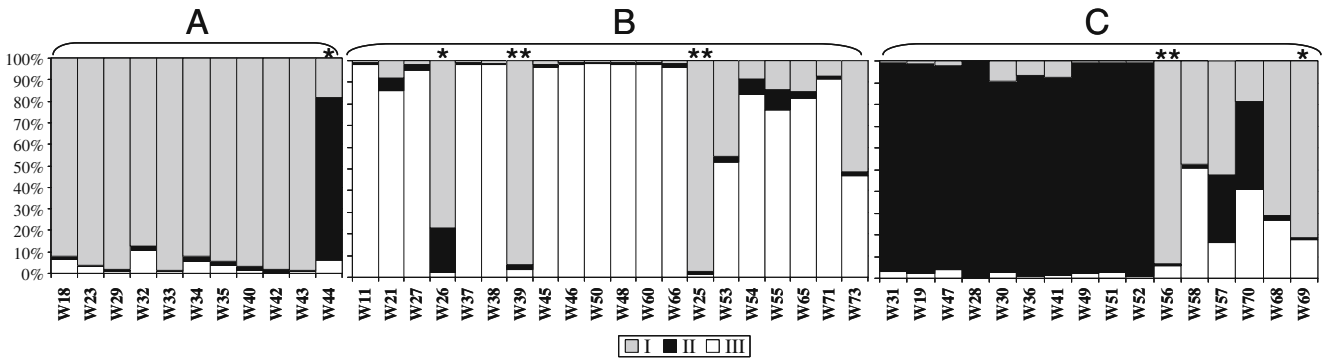

Fig. 5 Proportions of membership to the three inferred clusters, as resulting from the cluster analysis in STRUCTURE, for wolves sampled in areas *A*, *B*, and *C* (see Fig. 1) in the period 2000–2003. *Asterisks* indicate genotypes identified as immigrants, i.e. having a membership q to a cluster which is the most common in a different area (**q>90%; *q>75%)

data on wolf movements on a small geographic scale and the evidence of a cryptic genetic structure. We considered a recovered population that is central to the present wolf range in Italy and is likely to have been implied in the recolonization of the Western Alps (Scandura et al. 2001). It is distributed on contiguous mountain areas, where suitable habitats appeared to be saturated by wolf packs, with a seemingly high spatial stability (Capitani 2005; Capitani et al. 2006). Based on data obtained by Vonholdt et al. (2008) in Yellowstone National Park, we had predicted that a population with such characteristics as those observed in our study area (i.e. clumped and stable pack territories) should show high levels of genetic homogeneity, as a consequence of frequent interpack migration. On the basis of our results, this prediction can be rejected.

Temporal and spatial patterns, inferred by non-invasive resampling of genotyped individuals in the population, suggest indeed that the exchange of wolves between neighbouring packs is infrequent. Once being genotyped for the first time, a resampled wolf stood a good chance (approximately 80%) to be found in the same sampling unit. However, half of the wolves were not detected after their first sampling event. High mortality and high long-range dispersal rate can explain this low recapture rate, as most mortality events usually go unnoticed and long distance dispersers are likely to move out of the study area.

In agreement with resampling data, no evidence of short-range dispersal from the central AC pack was found during the period 2000–2003. During this period, reproduction in the AC pack by the genotyped dominant pair (male W11 and female W38) was confirmed every year and its summer rendezvous site was constantly recorded in the same small valley (Capitani et al. 2006). None of six possible offspring of this pair was sampled in more than 1 year and all but one occurred in the AC range only. The exception is represented by W48, who was found dead on the fringes of the AC pack territory but it was supposed to have been moved there after its death. Therefore, despite the central position and apparent stability of this pack, short-range migration of its members was not documented.

As expected, genetic relatedness decreased with increasing distance. Although two rivers separated areas A, B, and C (Fig. 1), they cannot represent effective barriers to wolf dispersal. Nevertheless, while wolves from adjacent SUs in the same area showed a moderate degree of relatedness, mean R dropped down to zero when it was calculated between wolves from neighbouring areas. Accordingly, a remarkable level of genetic differentiation among the three areas was found by means of Bayesian analysis. These results confirm the existence of constraints to local gene flow, with the two main valleys representing areas of genetic discontinuity. Nonetheless, between three (90% assignment criterion) and six (75% criterion) events of recent immigration were detected in the population.

Actually, pack stability can account for the maintenance of a remarkable degree of structuring in a population. Although mortality rate is not negligible in the study area, it seems to affect mainly young individuals, a general pattern observed in the rest of Italy as well (Capitani 2005; Lovari et al. 2007). This is also confirmed by the prevalence of this age class in our sample of dead individuals. In populations that are not characterized by the frequent loss of a breeder, breeding pairs can reproduce for several years and, if undisturbed, they can retain the same homesites and the same territories over time. This is for instance the case of the AC pack, as suggested by its summer locations (Capitani et al. 2006). Moreover, the local wolf population seems not only stable but also remarkably dense, with a close to saturation exploitation of suitable habitat (Capitani et al. 2006). This is a further factor promoting long distance migration in young wolves that may have difficulties finding a free suitable area within the Arezzo province

Being the Apennines a continuum of suitable habitats for wolves (Corsi et al. 1999), they can turn long-range dispersal into an effective strategy. The most striking evidence for this is the natural recolonization of the Western Alps, where

colonizing wolves were shown to have dispersed from the Northern Apennines (Scandura et al. 2001; Fabbri et al. 2007). The recent dispersal route of a radio-collared male (Ciucci et al. 2009) supports the idea of a general trend to long-range dispersal in the Italian population. Actually, long-range dispersal was reported to be common in wolves, and particularly frequent among young age classes, which can disperse over hundreds of kilometres (Mech and Boitani 2003). Dispersal distances may be very high in expanding populations, as observed in France (Valière et al. 2003) and Sweden (Vilà et al. 2002; Wabakken et al. 2007). In Finland, radiotelemetry and genetic data revealed that long-distance migrants represented a high proportion of dispersing individuals and their average dispersal distance was close to 100 km (Aspi et al. 2006; Kojola et al. 2006). Gula et al. (2009) documented a male wolf dispersing over a distance of 230 km in the Polish Carpathian Mountains, where no relevant genetic differentiation was detected.

If long dispersal distances can promote genetic homogenization on a wide geographic scale, this behaviour can limit exchange of individuals between close packs generating a hidden genetic structure, even in absence of physical barriers.

According to this hypothesis, areas like the one we monitored in the Arezzo province are likely to represent a source supplying migrants to the recolonization of peripheral sink areas. This interpretation can explain the ongoing expansion pattern in central Italy, where wolves are colonizing peripheral hilly areas and lowlands, after having completely recolonized mountain areas. Overall, our results suggest the existence of a cryptic genetic structure in the Apennine wolf population, which can result from a combination of demographic, social, and dispersal patterns, such as the stability of breeding pairs and the tendency to replace a breeder loss with individuals coming from afar (i.e. long-range dispersers, Smith et al. 1997).

Moreover, the present study demonstrates that the implementation of several opportunistic and non-invasive data sources to assess wolf movements and reconstruct genealogies in a population can provide valuable insights into the dynamics that influence genetic structure and demography in this species. Future studies, possibly employing both genetic and radiotelemetry data, are warranted to improve our understanding of the complex mechanisms that affect population dynamics in recovering South-European wolf populations.

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