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Abstract: We used noninvasive methods to obtain genetic and demographic data on the wolf packs (Canis lupus), which are now recolonizing the Alps, a century after their eradication. DNA samples, extracted from presumed wolf scats collected in the western Italian Alps (Piemonte), were genotyped to determine species and sex by sequencing parts of the mitochondrial DNA (mtDNA) control-region and ZFX/ZFY genes. Individual genotypes were identified by multilocus microsatellite analyses using a multiple tubes polymerase chain reaction (PCR). The performance of the laboratory protocols was affected by the age of samples. The quality of excremental DNA extracts was higher in samples freshly collected on snow in winter than in samples that were older or collected during summer. Preliminary mtDNA screening of all samples allowed species identification and was a good predictor of further PCR performances. Wolf, and not prey, DNA targets were preferentially amplified. Allelic dropout occurred more frequently than false alleles, but the probability of false homozygote determinations was always < 0.001. A panel of six to nine microsatellites would allow identification of individual wolf genotypes, also whether related, with a probability of identity of < 0.015. Genealogical relationships among individuals could be determined reliably if the number of candidate parents was 6-8, and most of them had been sampled and correctly genotyped. Genetic data indicate that colonizing Alpine wolves originate exclusively from the Italian source population and retain a high proportion of its genetic diversity. Spatial and temporal locations of individual genotypes, and kinship analyses, suggest that two distinct packs of closely related wolves, plus some unrelated individuals, ranged in the study areas. This is in agreement with field observations.
Noninvasive molecular tracking of colonizing wolf (Canis lupus) packs in the western Italian Alps

V. LUCCHINI,* E. FABBRI,* F. MARUCCO,† S. RICCI,† L. BOITANI‡ and E. RANDI*
Istituto Nazionale per la Fauna Selvatica (INFS), Ozzano Emilia (BO), Italy, †Progetto Lupo Interreg, Regione Piemonte, Parco Naturale Alpi Marittime, Valdieri (CN), Italy, ‡Dipartimento di Biologia Animale e dell’Uomo, Università ‘La Sapienza’, Roma, Italy

Abstract
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Keywords: Canis lupus, individual molecular tracking, microsatellites, molecular sexing, mtDNA control-region, noninvasive genetics

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Introduction
During the last two centuries wolf populations in Europe have declined strongly because of human persecution, deforestation and a decrease in their natural prey (Delibes 1990). Wolves were eradicated from the Alps in the 1920s and continued to decline in Italy until the 1970s, at which time ≈ 100 individuals survived in the central Apennines (Zimen & Boitani 1975). Legal protection, accorded in 1971, and active conservation contributed to the prevention of further population decline. From the 1980s onward wolves expanded along the Apennine ridge and recolonized parts of their historical range (Boitani 1992). Wolves crossed the north-western Apennines and reached the south-western Alps in 1992 (Breitenmoser 1998; Corsi et al. 1999; Poulle et al. 1999).

The genetic and demographic structure of the colonizing wolf population is unknown. Alpine wolves presumably originate from a natural expansion of the Italian source population. However, local shepherds and hunters claim that illegal releases of captive nonindigenous wolves occurred in parts of the study area.
wolves have contributed to the fast growth rate of the Alpine population (G. Canavese, personal communication). The current Alpine wolf population is small and population dynamics, interpack connectivity and dispersal rates of wolf packs cannot be estimated using only field observations.

Wolf colonization of the Alps represents an opportunity to apply noninvasive genetic methods (Kohn & Wayne 1997; Taberlet et al. 1997; Palsbøll 1999). DNA samples extracted from scats allow researchers to identify and map the presence of individual genotypes, and estimate their home ranges and relatedness (Palsbøll et al. 1997; Reed et al. 1997; Kohn et al. 1999; Woods et al. 1999; Ernest et al. 2000). However, the low amount and poor quality of target DNA extracted from noninvasive samples might produce contamination, allelic dropout or scoring of false alleles (Taberlet et al. 1996; Cagneux et al. 1997; Goossens et al. 1998; Miller et al. 2002). Therefore, the feasibility of any large-scale project should be assessed carefully through pilot studies (Taberlet et al. 1999).

Here we report the results of a pilot study aimed to explore the use of excremental DNA as a source of information for molecular tracking of local wolf packs. DNA samples were genotyped by: (i) nucleotide sequencing of the hypervariable part of the mitochondrial DNA control region (mtDNA CR), which is diagnostic for the Italian wolf population (Randi et al. 2000); (ii) analyses of restriction fragment length polymorphisms (RFLP) in sex-linked canine ZFX/ZFY DNA sequences (Garcia-Muro et al. 1997); (iii) genotyping of six to nine microsatellite loci (Fredholm & Winterø 1995; Francisco et al. 1996; Randi & Lucchini 2002). Genetic data were used to: (i) define the origin of colonizing Alpine wolves; (ii) estimate the number and relatedness of the different individual genotypes that were noninvasively sampled; (iii) map the spatiotemporal locations of the individual genotypes and wolf packs within the study areas, in comparison with observations that are being collected during ongoing field research.

Materials and methods

Sample collection

We studied two wolf packs, respectively, located in two areas separated by 40–50 km, in the Ligurian and Maritime Alps (Fig. 1). From May 1999 to August 2000 we collected 338 scats in the Ligurian Alps and 164 in the Maritime Alps, by following wolf snow tracks in winter and by travelling systematic transects along human trails/roads in summer. Scats were enclosed separately in clean plastic bags and stored at −18 °C until transferred to test tubes containing 95% ethanol. Before any further manipulation, all samples were deep-frozen at −80 °C for 10 days to kill any Echinococcus eggs.

These samples were not analysed randomly, but were selected on the basis of field observations to optimize the laboratory efforts and characterize the individuals in the packs. Wolves in the Ligurian Alps used an area of ≈ 300 km² (Valle Pesio, Valle Ellero, Val Corsaglia and other valleys; Fig. 1). We analysed scats collected mainly from Valle Pesio, where a rendezvous site was found in summer 1999, and where winter snow tracks suggested the simultaneous presence of several individuals. In summer 2000 wolf signs were seen mainly in Val Corsaglia where another rendezvous site was found 18 km from the summer 1999 site. Therefore, scats collected in Val Corsaglia from June to August 2000 were analysed to determine whether the same wolf pack used both areas and moved its rendezvous site to a new location in summer 2000. Samples collected in the Maritime Alps, from May 1999 to May 2000, were selected to locate individual wolves in the core area (the rendezvous site in Vallone del Piz) and in less frequently used peripheral areas (Vallone S. Anna and Vallone della Valletta; Fig. 1).

Rates of DNA degradation were evaluated in samples collected in ‘winter’ (November to April) and ‘summer’ (May to October). Scats were ranked as ‘fresh’ (1–2 days old when collected in summer, or 1–7 days old when collected on snow in winter), or ‘old’ (all the other samples), based on the time elapsed since the last sampling effort, scat appearance, exposure of deposition site and weather conditions (Ciucci et al. 1997). DNA quality is poor in aged scats; so samples older than two weeks were not analysed, except for those collected in Val Corsaglia and Valle Pesio in summer 1999, which were analysed anyway.

DNA analyses

DNA was extracted from ≈ 80 mg of scat material using a guanidinium thiocianate and diatomaceous earth protocol (Gerloff et al. 1995). Extractions were carried out in a room dedicated to excremental samples and all PCRs were performed under a laminar flow hood. Negative controls (no scat material added to the extractions) were used to check for contamination.

All DNA samples were initially screened by sequencing the diagnostic part of the mtDNA CR, which was amplified using primers l-Pro and H350 (Randi et al. 2000), with addition of 1 µg of BSA (Boehringer) to the PCR reactions and 40 amplification cycles. Sequences were obtained with the ABI (Foster City, USA) Dye-Terminator Kit and an ABI 373A automatic sequencer. ZFX/ZFY sequences (Garcia-Muro et al. 1997) were PCR-amplified in a volume of 25 µL using 10 µL of DNA solution, 2.5 µg of BSA and 40 PCR cycles. A sex-specific RFLP pattern was detected by overnight digestion of each PCR product with 10 units of HaeIII, and by 4% agarose gel electrophoresis in

TAE buffer. The ZFX product includes one HaeIII restriction site that produces two fragments of which only the larger (∼400 nucleotides) is visible on ethidium bromide-stained agarose gels. The HaeIII electrophoretic pattern is expected to show two visible bands in males (an uncut paternal ZFY fragment and a digested maternal ZFX fragment), but only one band in females (generated by digestion of both ZFX fragments).

Primers used for ZFX/ZFY amplifications (P1-5EZ and P2-3EZ; Aasen & Medrano 1990) are conserved in vertebrates and might amplify DNAs from wolf prey. Thus, we sequenced a subset of the PCR products including all the distinct individual multilocus genotypes. These sequences were aligned to homologous sequences from potential prey including roe deer (Capreolus capreolus), sheep (Ovis aries), pig (Sus scrofa) and horse (Equus caballus). Relationships among sequences and species assignment were obtained by neighbour-joining analysis (NJ; Saitou & Nei 1987) with Kimura 2-parameters (Kimura 1980) distance matrix and the software PAUP* Version 4.0b8 (Swofford 1998).

Nine loci, including six dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; Fredholm & Wintero 1995; and C09.250; Ostrander et al. 1993), and three tetranucleotides (FH2079, FH2088, FH2096; Francisco et al. 1996), were selected among 18 canine microsatellites previously used in a study of Italian wolves (Randi & Lucchini 2002). We used six microsatellites (CPH2, CPH8, CPH12, FH2079, FH2088, FH2096) to identify the genotypes, and we added three loci (CPH4, CPH5 and C09.250) to improve estimates of kinship. Microsatellites were PCR-amplified (Randi & Lucchini 2002) separately in 10 µL of volume, using 5 µL of DNA solution and 1 µg of BSA. Cycling conditions were optimized for each primer and the number of cycles varied from 40 to 45. These primers were also used to amplify DNAs from possible wolf prey. All microsatellites were analysed using an ABI 373A automatic sequencer and the ABI software GENOTYPER Version 2.1.

Fig. 1 Map of the two study areas in western Italian Alps, showing the geographical locations of wolf individual genotypes (W) in (a) the Maritime Alps and (b) the Ligurian Alps. Shaded areas indicate the approximate distributions of wolves in Italy.
Data analyses

Protocols for microsatellites were designed to implement the following multiple tubes approach (Taberlet et al. 1996; Gagneux et al. 1997): (i) all samples were amplified twice; (ii) those loci that were heterozygotes in both replicates were scored as reliable and genotypes were recorded; (iii) all homozygote and uncertain genotypes (due to failure of one amplification or to allelic dropout) were additionally replicated four times; (iv) all samples that could be not reliably typed at all loci after six amplifications were discarded. The overall occurrence of PCR uncertainty was computed using data from the repetition of 40 scat samples for each of the six loci (Table 2). The probability of false homozygotes was computed using the formula $P = (K/K/2)^n$ (Gagneux et al. 1997), where $K$ is the observed frequency of false homozygotes averaged over all samples and loci, and $n$ is the number of repeated amplifications.

The probability of different individuals sharing, by chance, an identical genotype (probability of identity, PID; Mills et al. 2000; Waits et al. 2001), and the expected PID among full sib dyads (PIDSibs), were estimated in a set of 100 Italian wolves using the software PROB-ID3 (G. Luikart unpublished). Field observations suggested that fewer than 10 wolves were present in each of the two study areas, so we aimed to achieve PIDSibs values $< 0.03$, meaning that 1 wolf in 30 siblings is expected to share, by chance, an identical genotype with another wolf. Wolves in a pack are known to be partially related, sharing alleles which are identical by descent (Mech 1970; Lehman et al. 1992; Wayne et al. 1995). Moreover, the colonizing population might be a nonrandom sample of the source Italian wolf population and different genotypes would differ in matching probability due to different allelic frequencies. Therefore, we computed individual match probabilities (Woods et al. 1999) for random and sibling individuals. Allelic frequencies were estimated from the Italian wolf population (Randi & Lucchini 2002).

Total match probabilities for each multilocus genotype were computed by multiplying single locus probabilities, assuming that loci are independent, as suggested by the microsatellite linkage map of the domestic dog (Neff et al. 1999).

Values of relatedness ($r$; Queller & Goodnight 1989) among genotypes were inferred using nine microsatellites, which were typed in a subset of DNA samples including all the different genotypes identified in the Alpine wolves, using the software KINSHIP Version 1.2 (Goodnight & Queller 1999). KINSHIP allows estimation of the likelihood and testing of the likelihood ratios of primary/null hypothetical pedigree relationships. In this study we tested a primary hypothesis of first-order relationship (i.e. dyads represent full sibs or parental-offspring with expected relatedness $r = 0.5$) vs. a null hypothesis of no relationship ($r = 0.0$). When likelihood ratios are significantly greater than 1, we can reject the null hypothesis and avoid a type II error (that siblings are incorrectly classified as unrelated) at a given level of significance. Equivalent microsatellite data from 100 Italian wolves were used to generate the expected distributions of pairwise relatedness among parents plus full sibs (theoretical average $r = 0.5$) and among unrelated individuals (theoretical average $r = 0.0$).

The proportion of parentages that can be theoretically identified with 80 or 95% confidence, given a set of specified parameters (number and proportion of typed loci, proportion of mistyped loci, number of candidate parents and proportion of candidate parents sampled), and using allele frequencies estimated in the source Italian wolf population, was simulated using CERVUS Version 1.0 (Marshall et al. 1998). Hypothetical full sib or parental–offspring dyads were re-examined using the likelihood approach implemented in CERVUS, which computes a log-likelihood of parentage (LOD score) for each candidate parent. CERVUS was also used to estimate the parameters of genetic diversity (number of alleles, expected $H_q$ and observed $H_o$ heterozygosity), and values of the polymorphic information content (PIC; Hearne et al. 1992) in the colonizing and source Italian wolf populations.

Departures from Hardy–Weinberg equilibrium (HWE) were tested using the probability test by Guo & Thompson (1992) as implemented in GENEPOP Version 3.2 (Raymond & Rousset 1995) and an estimate of $F_{is}$ (Weir & Cockerham 1984). The origin of the Alpine wolves was inferred by an assignment test of individuals to populations, using a Bayesian model implemented in the software STRUCTURE (Pritchard et al. 2000), and a set of 100 randomly sampled dogs, 100 Italian wolves and 170 wolves from Bulgaria, Croatia, Greece, Turkey, Israel, Latvia, Finland and Spain (unpublished).

Results

Age of the samples and performance of the laboratory protocols

We analysed 130 DNA samples extracted from 104 scats collected in the Ligurian Alps and 26 collected in the Maritime Alps. We obtained reliable mtDNA sequences in 84% of the cases, whereas 64 and 53% of the samples were successfully sexed and genotyped at all six microsatellites using the multitypes approach (Table 1). Thus, independent of the putative age and sampling period, the mtDNA CR amplified more frequently than nuclear genes ($P < 0.0002; \chi^2$ test). DNA samples extracted from scats collected in winter performed significantly better than those collected in summer ($P < 0.0001; \chi^2$ test). The mtDNA CR and microsatellites amplified better in DNAs extracted from fresh than from old samples ($P < 0.0001; \chi^2$ test). In contrast, amplification of the ZFX/ZFY sequences, which were used for sex determination, was not affected by the period of sampling or the age of the samples.
Rates of allelic dropout were different among loci, occurring in the 18% of the heterozygote determinations, on average (Table 2). The probability of false homozygote determinations after four positive PCR repetitions was \( P < 0.001 \). Contamination (i.e. the presence of more than two alleles in a sample) or false alleles (i.e. alleles falling outside the molecular mass range observed at each locus in the Italian wolves; Randi & Lucchini 2002; see Table 1), were detected in only five amplifications from different samples over >1500 PCRs performed in this study. These artefacts did not significantly affect the results.

The microsatellites used in this study did not amplify DNAs from ungulates, showing that prey DNAs did not affect genotype determinations. These primers amplify homologous loci from dogs and foxes (Vulpes vulpes), which, respectively, showed different allele frequencies or different allele sizes (Randi & Lucchini 2002). Thus, the assessment of allele size differences or an assignment test (see below) can be used to identify dog and fox samples.

**Mitochondrial sequences and species identification**

We obtained 109 mtDNA CR sequences from 130 analysed scats, which were identical to the Italian wolf haplotype W14 (Randi et al. 2000). Two sequences were identified as a dog and fox, respectively. The dog haplotype joined the dog cluster C1 (see Randi et al. 2000), and the domestic origin of this sample was confirmed by the assignment test of its microsatellite genotype. The identification of the fox sample was not obtained by the CR, which was difficult to sequence, but by sequencing part of the mitochondrial cytochrome \( b \) gene, and by comparing this sequences with DNA samples extracted from a set of fox tissues (not shown).

**Microsatellite variability and genotype identification**

Among the 69 scat samples which were successfully genotyped using six loci, we identified 14 distinct genotypes, which presumably correspond to at least 14 different individuals. In Valle Pesio we identified four males (W1, W2, W5 and W6) and three females (W3, W4 and W7). In Valle Corsaglia we identified two genotypes, identical (also in sex) to wolves W1 and W3 previously sampled in Valle Pesio. Wolves W1 and W3, sampled near the rendezvous site in 1999, were also sampled near the rendezvous site in 2000 (Fig. 1). This result supports field observations and suggests that the same wolf pack ranged in Valle Pesio and Val Corsaglia in 1999–2000, and moved between sites during summer 2000. In the Maritime Alps we identified seven new genotypes (W8, W9, W11, W12, W13, W14 and W15; Fig. 1), sampled from May 1999 to April 2000, which were different from those found in the Ligurian Alps (see Fig. 5). All the genotypes were assigned to the Italian wolf population using *structure*, with a probability \( q > 0.98 \), and had no significant ancestry in the dog gene pool or in other wolf populations.
Values of probability of identity observed (PIDobs) and expected in presence of siblings (PIDsibs), as related to the number of microsatellites typed in a sample of 100 Italian wolves, computed using six loci. The arrow indicates the PIDobs value obtained using six loci.

Recapture rates of genotypes varied in each study area. Wolves W1, W2, W3 and W4 were identified from 10 or more scats each; W5, W6 and W7 were identified from one or two scats. Wolf W12 was sampled eight times in the Maritime Alps, the other genotypes one or two times. These genotypes were not sampled during the same season. The highest proportion of new genotypes was observed in January and February 2000. In the Ligurian Alps, the most frequent genotypes (W1, W2, W3 and W4) occurred almost regularly from summer 1999 to spring 2000, except for W4 which was sampled regularly in Valle Pesio only from the end of July to December 1999. The other genotypes (W5, W6 and W7) were documented only in winter 2000. Only wolves W1 and W3 were resampled in summer 2000. In the Maritime Alps we found three different genotypes before winter (W9, W12, W13). All the other genotypes were found in January and February 2000.

The observed PID was zero, computed using up to five loci in a sample of 100 Italian wolves (Fig. 2). The theoretical PID for siblings was $1.50 \times 10^{-2}$ with six loci, suggesting that the selected microsatellites should allow detection of unique genotypes also if related individuals were sampled. Allele frequencies at each locus vary in the Italian wolf population (Randi & Lucchini 2002), and some genotypes are expected to be more frequent than others. However, match probabilities were usually $< 0.025$, which means that only 1 in 40 sibling individuals would share an identical genotype with another wolf by chance.

All microsatellites were polymorphic in the colonizing wolf population, showing 2–4 alleles (average = 2.67 $\pm$ 0.50). These loci were more variable in the Italian wolf population (3–7 alleles; average = 4.50 $\pm$ 1.38). Average values of gene diversity in the colonizing wolves ($H_3 = 0.524$, $H_E = 0.517$ and PIC = 0.414) were similar to the corresponding values in the source population ($H_3 = 0.650$, $H_E = 0.598$, and PIC = 0.542), suggesting that the colonizing population retains a high proportion of the genetic diversity of the source population. The colonizing population was in HWE (the probability to obtain by chance a value of $F_{st}$ greater than the observed was $P = 0.844$), whereas the Italian population was in disequilibrium ($P = 0.031$), likely due to geographical substructuring along the Apennines (see also Randi & Lucchini 2002).

Sex-linked sequences and molecular sexing of the colonizing wolves

DNA samples were sexed using diagnostic ZFX/ZFY RFLPs. We validated the sex-specific pattern in 20 sexed Italian wolves. Results of the molecular sexing procedure were 100% concordant with a priori known nonmolecular sexing. Using this procedure we identified 45 males and 41 females among the DNA samples collected in the Alpine study areas. However, these data should not be used to estimate the sex ratio of the colonizing wolf population, because the sampling probabilities of the different genotypes are very skewed. Among the 14 different individual microsatellite genotypes we identified 7 females and 7 males (sex ratio of 1:1).

The male-specific RFLP pattern allows recovery and sequencing of the ZFX and ZFY DNA fragments from agarose gels. The NJ tree (Fig. 3), representing the phylogenetic relationships among these sequences, showed that: (i) the average divergence between wolf ZFX and ZFY sequences was 0.033; (ii) wolf and dog ZFX and ZFY sequences joined into two distinct monophyletic clusters supported by bootstrap values $= 99\%$ (1000 bootstrap resamplings); (iii) prey ZFX and ZFY sequences joined into a distinct monophyletic cluster (bootstrap $= 71\%$). Thus, wolf and prey ZFX/ZFY sequences can be identified unambiguously. All sequences obtained from excremental DNAs were of wolf origin, and no prey ZFX/ZFY DNAs were amplified from the scats.

Inferring relatedness in the wolf packs

The simulated distributions of relatedness in unrelated (average $r = 0.002 \pm 0.250$) and parents plus full sibs (average $r = 0.476 \pm 0.220$) Italian wolves were partially

overlapping (Fig. 4). According to Blouin et al. (1996), the midpoint between the average of the two distributions (which in this case is 0.239) can be used as the cut-off value for the classification of the individuals. A dyad with \( r = 0.239 \) would be included in the distribution of unrelated individuals, whereas a dyad with \( r > 0.239 \) would belong to the parent–full sib distribution. The expected percentage of parent–offsprings or full sibs that would be misclassified as unrelated is 6.1 and 3.1%, respectively, and the expected percentage of unrelateds that would be misclassified as parents–full sibs is 17.1%. The average relatedness estimated in the wolf packs from the study areas (\( r = 0.498 \pm 0.199 \) in Ligurian Alps, and \( r = 0.252 \pm 0.402 \) in Maritime Alps) were higher than \( r \) obtained in unrelated Italian wolves.
showing that colonizing wolf packs likely include mainly related individuals.

Using cervus we simulated the proportion of parentage tests that can be resolved with 80 and 95% confidence using nine loci, an error rate of 1%, from 2 to 10 candidate parents, 100% of the loci were typed for each individual and the typing error rate was 1%.

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<th>Candidates parents</th>
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<th>One parent known</th>
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Table 3 Proportion of simulated parentage tests (10,000 simulations) resolved with 80 and 95% confidence estimated using cervus (Marshall et al. 1998). Allelic frequencies at nine microsatellite loci were determined in a sample of 100 Italian wolves. Simulations assumed that 100% of the loci were typed for each individual and the typing error rate was 1%.

A UPGMA clustering of interindividual relatedness showed that the wolf genotypes form two separate groups (Fig. 5): group A, including wolves W11, W12, W13 and W14 sampled in the Maritime Alps; group B, including wolves from the Ligurian Alps and W15 and W8 from the Maritime Alps. Wolves W7 (Ligurian Alps) and W9 (Maritime Alps) were not closely related to any other Alpine wolf. Groups A and B correspond in part to the packs mapped in the two study areas (Fig. 1).

Pedigree analyses were performed to test the primary hypothesis that dyads include full siblings (expected $r = 0.5$) vs. a null hypothesis of unrelatedness ($r = 0.0$). In the Ligurian Alps we identified highly significant relationships ($P < 0.01$) between wolves W1 and W2, and between W4 and W5. W1 was also related to W3, W4 and W5 ($P < 0.01$); W3 was also related to W6 ($P < 0.01$; Fig. 6). Closely related wolves W11, W12, W14 and W13 ($P < 0.01–0.001$), plus three unrelated wolves (W8, W9 and W15) were identified in the Maritime Alps. Wolves from the two study areas were not significantly related to each other except for W15 and W13 (sampled in the Maritime Alps), which were, respectively, related ($P < 0.01$) to W4–W5 and W3 (sampled in the Ligurian Alps). These data suggest that genotypes identified in the study areas should belong to two distinct packs.

LOD scores generated by cervus were used to identify the most likely parents in the two packs (Fig. 6). Wolves W1 and W3, sampled at least 10 times from June–July 1999 to spring 2000 in the Ligurian Alps, were the likely parents of W4, sampled 11 times from July to December 1999, and W5 and W6, sampled only in December 1999 and January 2000. Wolf W2, sampled 12 times from June 1999 to spring 2000, and W7 were significantly related, but they were not

identified as a likely parent–offspring dyad, and could be brothers.

Field observations suggested the presence of at least three wolves in the Maritime Alps, and in summer 1999 a core area was identified in Vallone del Piz where puppies were heard howling. Among the seven genotypes identified in the Maritime Alps, only W11, W12, W13 and W14 were significantly related. LOD scores suggested that male W12 (sampled eight times from July 1999 to March 2000) and female W13 (sampled two times, in September 1999 and January 2000), were the likely parents of W11, that was sampled only in January 2000, but not of W14. This wolf could be a brother of W12, as they appear to be closely related (Fig. 6).

Discussion

The recolonization of the Alps is of paramount importance for the conservation of the Italian wolf population, which through the Alps could reconnect to other populations, interrupting its long-lasting genetic isolation. Because of their elusive nature, direct monitoring of wolf packs in the Alps is problematic. Thus, the use of noninvasive molecular methods can add relevant information. However, the quality and power of noninvasive DNA data should be carefully evaluated to avoid pitfalls and optimize sampling procedures.

Preliminary mtDNA screening allows discarding of DNA samples that are too degraded for molecular sexing and microsatellite genotyping. Mitochondrial genes may be typed more easily than nuclear genes, because the mtDNA genome is present in multiple copies in cells and cellular extracts. Thus, diluted and degraded DNAs from excremental samples might limit the amplification of single-copy genes more than the highly repetitive mitochondrial genes (Kohn & Wayne 1997; Frantzen et al. 1998).

Species identification by mtDNA typing should be performed first, to avoid further analyses of wrong samples (e.g. dogs or foxes). DNA samples that did not amplify for mtDNA, also did not amplify for sex-linked or microsatellite loci. All the samples that were successfully genotyped with microsatellites also produced clear mtDNA sequences, whereas only 85% of them was successfully sexed. The ZFX/ZFY fragment is longer than microsatellites (≈ 430 nucleotides, whereas microsatellites range from 92 to 295 nucleotides), and is probably more sensitive to DNA degradation. We suggest targeting shorter fragments for sex determination, with a length comparable with that of microsatellites. In this study allelic dropout produced inaccurate genotyping in 18% of the PCR experiments, whereas amplification of false alleles was negligible. We observed a large per locus variance in dropout rates (Table 2), indicating that some loci are amplified less efficiently than others using excremental DNA samples. Inefficient
loci could be replaced, in future studies, with other loci, which should be equally informative, but less prone to allelic dropout. These findings support the use of the multiple tubes approach (Taberlet et al. 1996) to achieve the precision required for determining the individual genotypes. However, efficient multiple tubes strategies should be designed using a precise statistical framework, tailored to detect the most efficient loci and to direct the replications towards the problematic ones (Miller et al. 2002).

The presumed ‘age’ of samples and sampling ‘season’ significantly affected the performances of DNA analyses. Samples collected in winter performed generally better than samples collected in summer. However, obtaining noninvasive multilocus genotypes is still problematic and labour intensive. We suggest that field sampling strategies should be carefully planned in advance. In an Alpine habitat, excremental sampling in winter through snow tracking might be optimal. Frequent controls of transects in summer, at least once a week, would also improve the quality of laboratory results.

All sequenced DNAs showed the exclusive presence of the Italian wolf haplotype W14 (Randi et al. 2000). The assignment test also showed that all individual microsatellite genotypes identified in the Alps belong to the Italian wolf population. Thus, both mtDNA and nuclear markers suggest that the ongoing recolonization of the Alps is due to the natural expansion of the Italian wolf population. Occasional cross-breeding of Italian wolves with free-ranging dogs has been feared and documented in Italy (Boitani 1983; Randi & Lucchini 2002). Although the presence of dogs has been directly observed or documented by excremental DNA analyses, we did not identify any putative case of admixed wolf–dog genotype in the Alpine study areas. Moreover, the alleged presence of illegally released nonindigenous wolves was not documented by this data set. However, the genetic composition of the expanding Alpine wolf population should be monitored continuously, and eventual cross-breeding with dogs or presence of nonindigenous wolves should be promptly assessed.

Prey or contaminant DNA did not affect our laboratory protocols, thus molecular sexing, species and population assignment were reliable. The identification of a dog and a fox DNA using both mtDNA sequences and microsatellite alleles suggests that these two samples were erroneously identified as wolf scats. Alternatively, it is possible that wolf scats were marked by dog or fox urine. However, in this case, we suspect that true wolf mtDNA and/or microsatellite alleles would have been amplified and typed concomitantly with the contaminant dog and fox DNAs, leading to anomalous sequence or allelic patterns.

Values of genetic diversity in Alpine and Italian wolves were similar, suggesting that the colonizing population retained a relevant portion of the genetic variability of the source population (see also Forbes & Boyd 1996; which obtained similar results in a study of colonizing wolves in Montana). The smaller number of alleles in colonizing wolves could have been inflated by sample size differences, that is 100 Italian wolves vs. the 14 different genotypes that were identified in the study areas. However, the average relatedness in colonizing wolf packs was higher than among a sample of Italian wolves, indicating that colonizing wolf packs likely include mainly related individuals.

Six microsatellites were sufficient to achieve reliable identification of individual genotypes in the Alpine wolves, with PIDsibs < 0.015. We identified 14 distinct genotypes, with a sex ratio of 1:1. Individual genotypes showed values of match probability < 0.025, which is low enough to avoid randomly typing two identical genotypes in a sample of 40 siblings. Recapture rates were variable: a few genotypes were collected several times, whereas others were collected only once or twice. Genotypes sampled only once were mainly from scats collected in winter along wolf snow tracks. Following a pack travel route in the snow it is possible to collect scats of each individual aside from the individual marking behaviour. Instead, scats collected in summer along human trails/roads, were likely samples from dominant individuals that frequently mark the territory. These findings are consistent with wolf marking behaviour which affects the patterns of defecation on trails (Peters & Mech 1975; Vilà et al. 1994; but see Kohn et al. 1999 in the coyote).

Wolves sampled within each area were more related to each other than wolves between the two study areas, and were subdivided into two distinct groups of relatedness (Fig. 5), mostly concordant with sampling locations (Fig. 1) and with the inferred patterns of kinship (Fig. 6). Wolves in group A likely constitute a single pack including the two parents (male W12 and female W13) one offspring (female W11) and a relative (male W14). These results are concordant with field data: wolves W12 and W13 were sampled from July–September 1999 to winter 2000, and could be the candidates parents. Group B includes mainly wolves from the Ligurian Alps, but also wolves W15 and W8 from the Maritime Alps. Wolves W7 (Ligurian Alps) and W9 (Maritime Alps) were not closely related to any other wolf. Wolves W1, W2 and W3 were sampled from June 1999 and their movements in the study area (Fig. 1) suggested that they were adults and likely candidate parents. Wolves W1 and W3 were sampled together near the rendezvous site in Val Corsaglia (Fig. 1). Wolves W4 and W5 were sampled around the rendezvous site in 1999 (Fig. 1) and their small scat sizes suggest that they were pups. Thus, the results of kinship analyses are mostly concordant with field observations.

The genealogical relationships are preliminary and need to be reassessed when additional data are available. The
microsatellite data generated in this study might allow for detailed pedigree inference only if the number of candidate parents in the study area is small (fewer than 6–8), and all or most of them are sampled. The number of parents–offspring in a study area can be inferred approximately by field observations (snow tracks and wolf howling). However, the reliability of field data would be uncertain due to the unknown structure of packs, turnover rates and immigration of dispersing wolves into the study areas. Under these conditions, inferring kinship from genetic data might be problematic, mainly because of the unknown proportion of sampled and genotyped candidate parents (Marshall et al. 1998).

The typed microsatellites showed fewer alleles in the colonizing wolves than in the Italian wolf population. Moreover, the average relatedness in the Alpine wolf packs was higher than the average relatedness among unrelated Italian wolves thus showing that colonizing wolves likely include related individuals. The simulated distributions of relatedness among Italian wolves suggested 17.1% of unrelateds could be misclassified as parents–full sibs. Low allelic diversity and high relatedness might reduce the likelihood to identify the pedigree relationships in packs, also because it is usually difficult to discriminate parent–offspring from full sibs dyads (Marshall et al. 1998). These results suggest that increasing the number of loci or including loci with more alleles in the colonizing population would be beneficial.

Conclusions

The information obtained from these analyses should be considered preliminary and needs to be supported by additional genetic and field data. However, it is apparent that noninvasive genetic sampling methods can address issues that could not be addressed in any other way. The screening of a limited number of genetic markers produces information which can reliably be used to identify species, origin of local populations, identity of individuals, areas and movement of packs, as well as to hypothesize detailed genealogical relationships and describe the fine structure of wolf packs. An important prediction of this study is that a limited improvement of field sampling procedures, which should focus on collecting fresh scats in winter along wolf snow tracks, could allow significant improvement in the quality of the laboratory data. However, a variety of field sampling schemes could be defined accordingly to the kind of genetic data which one aims to obtain. Intensive sample collection, randomized across the entire wolf pack range and concentrated in short period (a few months), before and after the reproductive period, should be obtained to use noninvasive genetic data for population size estimations.

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