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Noninvasive sampling and genetic variability, pack structure, and dynamics in an expanding wolf population

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After centuries of population decline and range contraction, gray wolves (*Canis lupus*) are now expanding in Europe. Understanding wolf social structure and population dynamics and predicting their future range expansion is mandatory to design sound conservation strategies, but field monitoring methods are difficult or exceedingly expensive. Noninvasive genetic sampling offers unique opportunities for the reliable monitoring of wolf populations. We conducted a 9-year-long monitoring program in a large area (approximately 19,171 km^2) in northern Italy, aiming to identify individuals, estimate kinship, reconstruct packs, and describe their dynamics. Of 5,065 biological samples (99% scats), we genotyped and sexed 44% reliably using 12 unlinked autosomal microsatellites, 4 Y-linked microsatellites, and a diagnostic mitochondrial DNA control-region sequence. We identified 414 wolves, 88 dogs, and 16 wolf × dog hybrids. Wolves in the study area belonged to at least 42 packs. We reconstructed the genealogy of 26 packs. The mean pack size was 5.6 ± 2.4 *SD*, including adoptees, with a mean minimum pack home range of 74 km^2 ± 52 *SD*. We detected turnovers of breeding pairs in 19% of the packs. Reproductive wolves were unrelated and unrelated dispersers founded new packs, except for 1 pack founded by a brother–sister pair. We did not detect multiple breeding females in any packs. Overall, the population was not inbred. We found significant isolation by distance and spatial autocorrelation, with nonrandom genetic structure up to a distance of approximately 17 km. We detected 37 dispersers, 14 of which became breeders in new or already existing packs. Our results can be used to model habitat use by wolves, to estimate survival rates, to predict future expansion of the wolf population, and to build risk maps of wolf–human conflicts.

Key words: *Canis lupus*, conservation genetics, gray wolf, inbreeding, kinship, noninvasive genetic sampling, pack structure, pedigree

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The development of noninvasive genetic methods has offered unique opportunities to implement long-term, wide-ranging, and cost-effective research and monitoring programs (Schwartz et al. 2007; Brøseth et al. 2010; Ruiz-González et al. 2013). Molecular techniques can provide more-exhaustive demographic information than any other method (Lukacs et al. 2007). Reliable individual genotypes (DNA fingerprinting) are obtained by analyzing DNA extracted from biological samples such as hair, feces, urine, and blood traces that are noninvasively collected, without any direct human contact with the animals (Waits and Paetkau 2005). Genotypes are used to count and locate individuals in space and time and to reconstruct their genealogies and familial ranges (Creel et al. 2003; Schwartz et al. 2007). The capture–recapture records of individual genotypes can be used to count the minimum population size (Ernest et al. 2000; Lucchini et al. 2002; Gervasi et al. 2008) and to estimate total abundance (Kohn et al. 1999; Mills et al. 2000; Lukacs and Burnham 2005). Although low-quality DNA samples may generate genotyping errors (Broquet et al. 2007), these can be minimized by using well-tested laboratory protocols and quality controls (Beja-Pereira et al. 2009). Noninvasive genetics has been used to

monitor the dynamics of endangered populations, obtaining estimates of temporal trends of demographic and genetic parameters that would have been impossible with traditional field methods (e.g., De Barba et al. 2010). The reconstruction of pedigrees in natural populations (Pemberton 2008) is facilitated by genetic identifications, which substantially help to infer detailed population structuring, and to estimate dispersal rates, inbreeding, and heritability (vonHoldt et al. 2008), pushing the development of novel computational methods (Blouin 2003). For these reasons, noninvasive genetic sampling has been integrated into many monitoring projects, combining population genetics and demographic data in species of large carnivores (Waits and Paetkau 2005), including studies of wolves (*Canis lupus*—Fabbri et al. 2007; vonHoldt et al. 2008; Marucco et al. 2009; Cubaynes et al. 2010; Stenglein et al. 2011).

Wolves offer interesting case studies of both theoretical and applied conservation biology (vonHoldt et al. 2008, 2013; Coulson et al. 2011; Wayne and Hedrick 2011). After centuries of population decline, wolves are now increasing in number and range size in North American and European countries (Leonard et al. 2005; Randi 2011). Expanding populations also spread in human-dominated areas, where they affect populations of wild and domestic ungulates (Boitani et al. 2010) and where the chances of hybridization with domestic dogs may increase (Verardi et al. 2006; Godinho et al. 2011; Hindrikson et al. 2012; vonHoldt et al. 2013). Long-term conservation of viable wolf populations entails solving both biological and human-dimension problems (Ciucci et al. 2007; Linnell and Boitani 2012). Thus, appropriate conservation projects and management strategies must be established, based on sound information on wolf biology and ecology (Chapron et al. 2003; Smith et al. 2003).

Most wolves are territorial, social carnivores that live in packs, the basal family units, which generally include a breeding pair, the offspring from several years, and sometimes unrelated wolves (Mech 1999). Packs scent mark and defend their territories, and territories often remain stable for several successive breeding pairs. Pack members cooperate in hunting and rearing pups (Mech and Boitani 2003). Pack size and composition, prey abundance, and habitat availability determine the demographic trends of wolf populations (Fuller et al. 2003; Stahler et al. 2013). In turn, variable mating behaviors, turnover rates of pack breeders, dispersal patterns, and interpack gene flow affect population genetic structure and long-term evolutionary dynamics (Lehman et al. 1992; Lucchini et al. 2004; vonHoldt et al. 2008; Sastre et al. 2011; Czarnomska et al. 2013). In this way, pack dynamics, natural selection, adaptation, and inbreeding avoidance affect kin structure and inbreeding and determine the evolution of genetic variability (Keller and Waller 2002; Bensch et al. 2006; Coulson et al. 2011; Geffen et al. 2011).

Determining wolf population structure and dynamics, however, is not trivial (Duchamp et al. 2012). Wolves are distributed at low densities across large geographic areas, often in forested mountain regions, and their individual and familial home ranges are wide (Jędrzejewski et al. 2007). In these conditions, standard field methods based on direct observations, livetrapping and radiotelemetry, snow-tracking, and distance sampling (Wilson and Delahay 2001; Meijer et al. 2008; Blanco and Cortés 2012) are challenging or exceedingly expensive at a large scale (Boitani et al. 2012; Galaverni et al. 2012). Consequently, most of the published studies report details based on short-term, empirical studies (i.e., Scandura et al. 2011). The result is that values of crucial demographic parameters such as survival, abundance, turnover, dispersal, and reproduction rates remain poorly known (Mech and Boitani 2003).

Here we summarize the results of a 9-year noninvasive genetic monitoring project in a wolf population that recently recolonized the Apennine Mountains of northern Italy (Caniglia et al. 2010, 2012). We designed our research to determine the genetic variability and integrity of the population, which might have been threatened by reduced effective size and hybridization with domestic dogs (Randi 2011); the number of packs (Mech and Boitani 2003); the size of the packs, including the number of unrelated (adoptee) wolves (Jędrzejewski et al. 2005); the relatedness of individuals in the packs and the frequency of inbred reproductive pairs (Lehman et al. 1992; vonHoldt et al. 2008); and the frequency of pack splitting during the process of population expansion (Jędrzejewski et al. 2005). Based on the territorial and hierarchical organization of wolf populations (Mech 1999), we reconstructed location and composition of the wolf packs predicting that dominant individuals would be sampled within defined geographic ranges (corresponding to their territories—Fuller et al. 2003); distinct packs would have nonoverlapping ranges, thus dominants from distinct packs would be sampled in nonoverlapping areas (Apollonio et al. 2004; Kusak et al. 2005, vonHoldt et al. 2008); dominants would mark their territories with scats and urine (Zub et al. 2003; Barja et al. 2005), so they would be sampled more frequently than young or transient individuals; breeding pairs should reproduce for at least 1 breeding season, and consequently would be sampled longer than young or transients (Mech and Boitani 2003); and pedigrees of familial groups could be reconstructed, given the power of the molecular markers used for genotyping (Pemberton 2008). Our results clarify details of wolf social behavior and wolf population dynamics in an area with diverse habitats and prey availability, and provide the basis necessary to forecast future demographic trends and ecological roles of wolves in northern Italy.

Materials and Methods

The study area.—Our study area was in the northern Apennine Mountains between Emilia Romagna and Tuscany (44°45′00″N, 11°00′00″E), covering a total area of about 19,171 km^2 (Fig. 1). Most of this area (70%) was above 700 m above sea level, with the highest peak, Mount Cimone, reaching 2,165 m above sea level. The vegetation was mainly temperate and sub-Mediterranean deciduous forests, densely

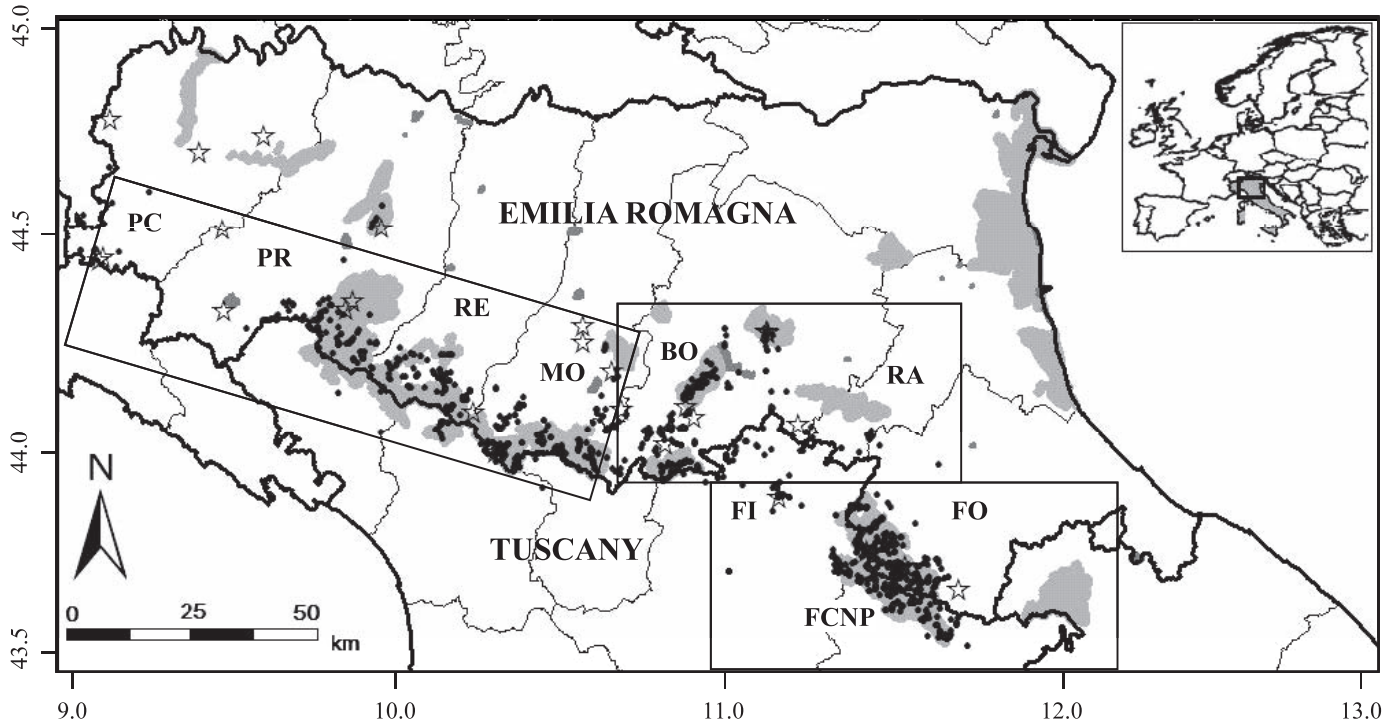

Fig. 1.—The wolf (*Canis lupus*) study area in the Emilia Romagna and Tuscany Apennines in Italy, with locations of the noninvasive wolf samples (filled circles) and wolves found dead (stars). The protected areas are in gray. Rectangles indicate the 3 main sectors of the study area. The eastern sector includes: FI = Florence Province, FO = Forlì-Cesena Province, and FCNP = Foreste Casentinesi National Park. The central sector includes: RA = Ravenna Province, and BO = Bologna Provinces. The western sector includes: MO = Modena Province, RE = Reggio Emilia Province, PR = Parma Province, and PC = Piacenza Province. Longitude and latitude are indicated on the x- and y-axes in decimal degrees (datum WGS84).

populated by wild ungulates, including wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*). The climate showed sharp seasonal changes with short periods of snowfall in winter. Temperature averaged 11°C (mean winter minimum –1°C; mean summer maximum 23°C). Mean rainfall was 769 mm (exceeding 1,500 mm in the Apennines ridge), concentrated in spring and autumn (ARPA 2010). Low elevations were characterized by pastures and livestock breeding, whereas slopes and valleys are cultivated. Some parts of the region are urbanized, with medium- to small-sized villages and a fairly dense road network. About 25% of the study area was protected in 2 national and 11 local parks (Fig. 1). The presence of free-ranging dogs has been repeatedly documented (Galaverni et al. 2012), but no estimates of their abundance are available.

Sampling.—Samples were collected from March 2000 to June 2009 by more than 150 trained collaborators, including staff of the Italian State Forestry Corps, park rangers, wildlife managers, researchers, students, and volunteers. Although the external appearance of scats might not reflect their age (Santini et al. 2007), collectors were trained to collect samples as fresh-looking as possible, excluding the most degraded ones. Feces were collected along a total of approximately 160 trails or country roads averaging about 6.1 km in length. Roads and trails were chosen opportunistically based on known or predicted wolf presence, as assessed by field surveys of wolf trails and snow tracks, documented kills, wolf-howling, or occasional direct observations, approximately covering the entire range of stable wolf distribution in the study area. Roads and trails were surveyed at least once per month, on average, either on foot or by car. Samples of muscle tissue were obtained from wolves killed accidentally or illegally. Blood samples were occasionally obtained during rescuing operations on wolves wounded or in poor healthy condition. Fecal sample collection did not require any direct interaction with the animals. We obtained the tissue samples from found-dead wolves legally collected by officers on behalf of the Italian Institute for Environmental Protection and Research (Istituto Superiore per la Protezione e la Ricerca Ambientale). No animal was sacrificed for the purposes of this study. Blood samples were obtained from rescued animals by appropriately trained veterinary personnel. Anesthesia was used whenever necessary to minimize any stress on the animals during handling procedures. All the procedures followed guidelines approved by the American Society of Mammalogists (Sikes et al. 2011). The coordinates of every sample (Fig. 1) were recorded either on a 1:25,000 topographic map or by global positioning system devices, then digitalized on ArcGis 10.0 (ESRI, Redlands, California).

The large study area and long-term program did not allow us to standardize or randomize sampling in space and time. Nevertheless, as highlighted in Jędrzejewski et al. (2008), heterogeneity should not bias the results in any systematic way. Small external portions of scats and clean tissue fragments were individually stored at –20°C in 10 vials of 95% ethanol. Blood samples were stored at –20°C in 2 vials of a Tris–sodium dodecyl sulfate buffer. DNA was automatically extracted using a MULTIPROBE II^{EX} Robotic Liquid Handling System (Perkin Elmer, Weiterstadt, Germany) and QIAGEN QIAmp DNA stool or DNeasy tissue extraction kits (Qiagen Inc., Hilden, Germany).

All the individual genotypes were assigned to their population of origin using 168 reference wolf genotypes (76 females and 92 males, randomly selected from wolves found dead in the last 20 years across the entire wolf distribution in Italy). All these animals showed the typical Italian wolf coat color pattern and neither morphologically nor genetically detectable signs of hybridization (Randi 2008). We also used a panel of reference dog genotypes from 115 blood samples randomly selected from wolf-sized dogs (50 females and 65 males) living in rural areas in Italy.

Laboratory methods.—We identified individual genotypes for samples at 12 unlinked autosomal canine microsatellites (short tandem repeats [STR]): 7 dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12, C09.250, and C20.253) and 5 tetranucleotides (FH2004, FH2079, FH2088, FH2096, and FH2137), selected for their high polymorphism and reliable scorability for wolves and dogs. We determined sex of samples using a polymerase chain reaction (PCR)–restriction fragment length polymorphism assay of diagnostic ZFX/ZFY gene sequences (Caniglia et al. 2012, 2013, and references therein). We used a panel of 6 STR to identify the genotypes with Hardy–Weinberg probability-of-identity (PID) among unrelated individuals, $PID = 8.2 \times 10^{-6}$, and expected full-siblings, $PID_{sibs} = 7.3 \times 10^{-3}$ (Mills et al. 2000; Waits et al. 2001) in the reference Italian wolves. We used another panel of 6 STR, also selected for their polymorphism and reliable scorability, to increase the power of admixture and kinship analyses, decreasing the PID values to $PID = 7.7 \times 10^{-9}$ and $PID_{sibs} = 3.1 \times 10^{-4}$ (Supporting Information S1, DOI: 10.1644/13-MAMM-A-039.S1). We identified maternal haplotypes by sequencing 350 base pairs of the mitochondrial DNA (mtDNA) control region, diagnostic for the haplotype W14, which is unique to the Italian wolf population, using primers L-pro and H350 (Randi et al. 2000). We identified paternal haplotypes by typing 4 Y-linked microsatellites (Y-STR), MS34A, MS34B, MSY41A, and MS41B (Sundqvist et al. 2001), characterized by distinct allele frequencies in dogs and wolves (Iacolina et al. 2010).

We amplified autosomal and Y-linked STR loci in 7 multiplexed primer mixes using the QIAGEN Multiplex PCR Kit (Qiagen Inc.), a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, California), and the following thermal profile: 94°C for 15 min, 94°C for 30 s, 57°C for 90 s, 72°C for 60 s (40 cycles for scat, urine, and hair samples, and 35 cycles for muscle and blood samples), followed by a final extension step of 72°C for 10 min. We carried out amplifications in 10-µl volumes including 2 µl of DNA extraction solutions from scat, urine, and hair samples, 1 µl from muscle or blood samples (corresponding to approximately 20–40 ng of DNA), 5 µl of QIAGEN Multiplex PCR Kit, 1 µl of QIAGEN Q solution (Qiagen Inc.), 0.4 µM deoxynucleotide triphosphates, from 0.1 to 0.4 µl of 10 µM primer mix (forward and reverse), and RNase-free water up to the final volume. We amplified the mtDNA control region in a 10-µl PCR, including 1 or 2 µl of DNA solution, 0.3 pmol of the primers L-Pro and H350, using the following thermal profile: 94°C for 2 min, 94°C for 15 s, 55°C for 15 s, 72°C for 30 s (40 cycles), followed by a final extension of 72°C for 5 min. PCR products were purified using exonuclease/shrimp alkaline phosphatase (Exo-Sap; Amersham, Freiburg, Germany) and sequenced in both directions using the Applied Biosystems Big Dye Terminator kit (Applied Biosystems, Foster City, California) with the following steps: 96°C for 10 s, 55°C for 5 s, and 60°C for 4 min of final extension (25 cycles). DNA from scat, urine, and hair samples was extracted, amplified, and genotyped in separate rooms reserved to low-template DNA samples, under sterile ultraviolet laminar flow hoods, following a multiple-tube protocol (Caniglia et al. 2012; 2013; for details, see Supporting Information S1 and S2, DOI: 10.1644/13-MAMM-A-039.S2), including both negative and positive controls. We obtained genotypes from blood and muscle DNA, replicating the analyses twice. DNA sequences and microsatellites were analyzed in a 3130XL ABI automated sequencer (Applied Biosystems), using the ABI software SeqScape 2.5 for sequences, and Genemapper 4.0 for microsatellites (Applied Biosystems). We report detection of PCR errors, mismatch analyses, postprocess quality-control procedures, and other laboratory details in Supporting Information S2–S4 (DOI: 10.1644/13-MAMM-A-039.S3 and DOI: 10.1644/13-MAMM-A-039.S4).

Population structure, assignment, and identification of wolf × dog admixed genotypes.—We assigned individual genotypes to their population of origin (wolves or dogs) using Structure 2.3 (Falush et al. 2003). We ran Structure with 5 replicates of 10^4 burn-in followed by 10^5 iterations of the Markov chain Monte Carlo sampling, selecting the "admixture" model (each individual may have ancestry in more than 1 parental population), either assuming independent or correlated allele frequencies. We identified the optimal number of populations K using the ΔK procedure (Evanno et al. 2005). At the optimal K we assessed the average proportion of membership (Q_i) of the sampled populations (wolves or dogs) to the inferred clusters. We assigned genotypes to the Italian wolf or dog clusters at threshold $q_i = 95$ (individual proportion of membership—Randi 2008), or identified them as admixed if their q_i values were intermediate. We checked putative wolf × dog hybrids further using additional admixture analyses on observed and simulated genotypes obtained by HybridLab (Nielsen et al. 2006) and using diagnostic mtDNA and Y-STR haplotypes.

Genetic variability.—Based on the assignment tests, we grouped all genotypes as those of wolves, dogs, or hybrids. We used GenAlEx 6.1 (Peakall and Smouse 2006) to estimate allele frequency by locus and group, observed (H_O) and expected unbiased (H_E) heterozygosity, mean (N_A) and expected (N_E) number of alleles per locus, number of private alleles, and PID and PID_{sibs}. We calculated the polymorphic information content (PIC) using Cervus 3.0.3 (Kalinowski et al. 2007). We computed Wright's inbreeding estimator (F_{IS}—Weir and Cockerham 1984) and departures from Hardy–Weinberg equilibrium using Genetix 4.05 (Belkhir et al. 1996–2004). We assessed F_{IS} significance using 10,000 random permutations of alleles in each population. We tested for the occurrence of null alleles in Microchecker (Van Oosterhout et al. 2004). We estimated inbreeding coefficient F of Lynch and Ritland (1999) using Coancestry 1.0 (Wang 2011), with allele frequencies and PCR error rates assessed from the sampled population and 95% confidence intervals (*CI*s) generated though 1,000 bootstrapped simulations. We used the sequential Bonferroni correction test for multiple comparisons to adjust significance levels for every analysis (Rice 1989). Throughout the paper, we express estimates of variability as the mean ± *SD*.

Identification of packs, pedigrees, and dispersal.—We selected all the genotypes that were sampled in restricted ranges (< 100 km^2) at least 4 times and for periods longer than 24 months. We determined their spatial distributions by 95% kernel analysis, choosing band width using the least-squares cross-validation method (Seaman et al. 1999; Kernohan et al. 2001), using the AdehabitatHR package for R (Calenge 2006) and mapped them using ArcGis 10.0. According to spatial overlaps, we split individuals into distinct groups that might correspond to packs, for which we performed parentage analyses. We reconstructed the complete genealogy of each group using a maximum-likelihood approach implemented in Colony 2.0 (Wang and Santure 2009). For each area, we considered as candidate parents all the individuals sampled in the 1st year of sampling and more than 4 times in the same area and as candidate offspring all the individuals collected within the 95% kernel spatial distribution of each pack and in a surrounding buffer area of approximately 17-km radius from the kernel (see "Results"). We ran Colony with allele frequencies and PCR error rates as estimated from all the genotypes, assuming a 0.5 probability of including fathers and mothers in the candidate parental pairs. To be sure that all the possible parentages were detected, we compared the best maximum-likelihood genealogies to those obtained by an "open parentage analysis" in Colony, using all the males and females as candidate parents, and all the wolves sampled in the study area as candidate offspring. We also compared the best maximum-likelihood genealogies reconstructed by Colony with those obtained by a likelihood approach in Cervus, based on the Mendelian inheritance of the alleles, accepting only parent–offspring combinations with at most one-twenty-fourth allele incompatibilities, and father–son combinations with no incongruities at Y-STR haplotypes. We determined parentage assignments in Cervus using natural log of likelihood ratio scores for candidate parents, given the set of candidate offspring genotypes and the allele frequencies in the whole population (when a natural log of likelihood ratio score was positive, the candidate parent is the most likely true parent [Kalinowski et al. 2007]). We also performed simulations to determine the likelihood of randomly selected parents. We considered natural log of likelihood ratio values that were significant at 95% and 80% thresholds. Natural log of likelihood ratio scores were generated by simulating 10,000 offspring and 50 candidate males, allowing for 20% of the population to be unsampled, 20% incomplete multilocus genotypes, and the genotyping error rate as empirically estimated from the data set (vonHoldt et al. 2008).

We estimated values of relatedness (r—Queller and Goodnight 1989) within and among packs using KinGroup 2.0 (Konovalov et al. 2004) and compared those with values of 1st-order (parent–offspring plus full siblings) and unrelated dyads estimated from 1,000 simulated pairs. We used a likelihood ratio test with a primary hypothesis of $r = 0.25$ (half siblings or cousins) and $r = 0.50$ (full-siblings or parent–offspring) versus a null hypothesis of $r = 0.00$ (unrelated) to test for inbreeding within and among packs, at the $\alpha = 0.05$ level.

We used locations of individuals in the packs in ArcGis 10.0 to reconstruct the areas and centroids of the 95% kernel spatial distribution for each pack, and the distances between centroids; reconstruct the minimum, median, and maximum distance of genotypes to the pack centroids; and identify dispersing wolves. We identified individuals sequentially sampled in different territories (> 17 km apart), or that reproduced in a pack different from their natal one, as putative dispersers. We considered individuals that were not assigned to a pack and the dispersers that did not establish in any pack as potential floaters.

Spatial analyses.—We assessed fine-scale spatial genetic structure by multivariate spatial autocorrelation analyses of geographical and genetic distances in SPAGeDI 1.2 (Hardy and Vekemans 2002) and estimated through the autocorrelation kinship coefficient F_{ij} (Loiselle et al. 1995), which is similar to Moran's I (Smouse and Peakall 1999) but is relatively unbiased even with low sampling variance. We calculated F_{ij} for distance classes that had been determined based on wolves' home ranges and following recommendations of Hardy and Vekemans (2002). Thus, we used the equal frequency method, which assumes that more than 50% of all individuals were represented at least once in each spatial interval. We tested the 95% F_{ij} *CI*s and the nonrandom spatial genetic structure via 10,000 permutations and we investigated the effects of behavioral biases (sex-biased dispersal and pack relatedness) by computing autocorrelations separately in males, females, and breeding pairs. We computed correlations between geographic and genetic distance of individuals and packs after permuting the locations, similarly to a Mantel test (Mantel 1967). Whenever possible, we used additional field information such as snow-tracking, wolf-howling, camera

trapping, and occasional direct observations to evaluate the reliability of the inferred pack structure and locations.

Results

Sampling.—We collected 5,065 samples including 4,998 scats, 4 hair tufts, 2 urine stains found during snow-tracking, 57 samples of muscle tissue obtained from wolves killed accidentally or illegally, and 4 blood samples obtained from livetrapped wolves. More feces were collected in autumn and winter (72.3%) than in spring and summer. The average number of samples per year was 562.8 ± 334.7 for the entire study area, and 234.9 ± 174.2, 146.6 ± 101.4, and 160.9 ± 53.2 in the eastern, central, and western sectors, respectively

Identification and assignment of the individual multilocus genotypes.—The multiple-tube PCR and mismatch analyses, and post-PCR controls identified 480 distinct reliable genotypes ($R \geq 0.95$) corresponding to 2,202 (44%) of the total 5,004 noninvasive DNA samples collected in the study area (Supporting Information S3 and S4). The 61 muscle and blood samples yielded 56 (92%) reliable and distinct genotypes. Eighteen of them matched with genotypes obtained from noninvasive samples, and 38 were never sampled before. All the 518 distinct genotypes were assigned to their population of origin at $K = 2$, which showed the maximum ΔK value ($\Delta K\ 2 =$ 2,230.59; $\Delta K\ 3 = 36.01$; $\Delta K > 3 \leq 22.93$). All reference wolves were assigned to 1 cluster (w) with $Q_w = 1.00$ (individual q_w ranging from 0.99 to 1.00) and all reference dogs were assigned to the other cluster (d) with $Q_d = 0.99$ (individual q_d ranging from 0.95 to 1.00). At threshold $q_w = 0.95$ (which was supported also by the assignments of HybridLab-simulated genotypes; data not shown), the genotypes with $0.05 \leq q_w \leq 0.95$ were considered as admixed (Table 1). Thus, 414 (80%) of the 518 new genotypes were assigned to the wolf cluster ($q_w > 0.95$), 88 (16%) were assigned to the dog cluster ($q_d > 0.95$), and 16 (4%) were partially assigned to both clusters with $0.73 < q_w < 0.94$ (wolf × dog admixed genotypes; Table 1).

Genetic variability in the wolf population.—All microsatellites were polymorphic in the 414 wolves sampled in the study area. The 16 hybrids were excluded from these analyses to avoid the risk that alleles from dogs inflate the genetic variability of the wolf population. Wolves showed from 2 to 11 alleles (average $N_A = 5.25 \pm 2.29$ in wolves in the study area, and $N_A = 4.50 \pm 2.08$ in reference wolves; significantly different, $t_{11} = 3.00$, $P = 0.01$; *t*-test), and intermediate values of heterozygosity ($H_O = 0.56$, $H_E = 0.58$, PIC = 0.52 in wolves in the study area; $H_O = 0.55$, $H_E = 0.58$, PIC = 0.53 in reference wolves; not significantly different, $t_{11} = 1.18$, $P = 0.26$ for H_O; $t_{11} = 0.78$, $P = 0.45$ for H_E; $t_{11} = 0.88$, $P = 0.39$ for PIC; *t*-tests). Microsatellite loci were not significantly out of Hardy–Weinberg equilibrium in wolves in the study area, showing a slightly positive, but nonsignificant F_{IS} value (0.037 ± 0.090; $P = 0.35$; Table 2). In contrast, reference dogs and wolves were not in Hardy–Weinberg equilibrium due to fewer observed than expected heterozygotes (significantly positive F_{IS}; Table 2; Supporting Information S5, DOI: 10.1644/13-MAMM-A-039.S5).

Table 1.—Sample size and summary of genetic identifications obtained by genotyping 12 autosomal microsatellites (short tandem repeats [STR]), 4 Y-linked STR, and the mitochondrial DNA (mtDNA) control region in reference wolves (*Canis lupus*), wolves sampled in the study area, dogs, and wolf × dog hybrids. *N* total = number of distinct genotypes (number of males [no. males]); Q_w and Q_d = proportions of membership of each group to the wolf or dog cluster, respectively, in an admixture analysis with $K = 2$ (Structure—Falush et al. 2003); W14 = frequency of the diagnostic Italian wolf W14 mtDNA control-region haplotype; Y-STR = number and frequency of the Y-STR haplotypes as named by Caniglia et al. (C; 2010), Sundqvist et al. (S; 2001), and Iacolina et al. (I; 2010).

Group	*N* total (no. males)	Q_w	Q_d	W14	Y-STR$_C$	Y-STR$_S$	Y-STR$_I$
Reference wolves	168 (92)	1.00	0.00	100%	U (72; 79%)	—	H1
					I (17; 18%)	Q	H2
					D (3; 3%)	—	—
Wolves in the study area	414 (236)	1.00	0.00	100%	U (195; 82%)	—	—
					I (28; 12%)	Q	—
					L (13; 6%)	L	—
Reference dogs	115 (65)	0.01	0.99	0%	L (23; 35%)	L	H3
					D (17; 26%)	—	—
					O (5; 8%)	—	—
					C (3; 5%)	—	—
					Q (3; 5%)	—	—
					V (3; 5%)	—	—
					S (2; 3%)	—	—
					T (2; 3%)	G	—
					Y (2; 3%)	—	—
					E (1; 2%)	—	—
					K (1; 2%)	—	—
					N (1; 2%)	—	—
					P (1; 2%)	C	—
					R (1; 2%)	—	—
Dogs in the study area	88 (42)	0.01	0.99	0%	L (16; 38%)	L	H3
					D (13; 31%)	—	—
					P (3; 7%)	C	—
					4 (2; 5%)	—	—
					M (2; 5%)	—	—
					J (2; 5%)	—	—
					F (1; 4%)	—	—
					O (1; 2%)	—	—
					Z (1; 2%)	—	—
Hybrids	16 (11)	0.83	0.17	100%	U (6; 55%)	—	H1
					P (2; 18%)	C	—
					O (1; 9%)	—	—
					1 (2; 18%)	J	H4

In the study area, 236 wolves were males and 178 females (sex ratio male : female = 1.3, significantly different from 1; $\chi^2_1 = 8.12$, $P < 0.001$; chi-square test). All wolves showed the diagnostic W14 mtDNA control-region haplotype (Randi et al. 2000), which was absent in dogs. Overall, we identified 22 Y-STR haplotypes (Table 1), which were differently distributed in wolves (4 haplotypes, of which 2 were unique) and dogs (19 haplotypes, 15 unique). The most frequent wolf haplotypes, U and I, occurred in 223 (94%) males in the study area and 89 (97%) reference wolves, and were absent in dogs. The most

Table 2.—Genetic variability at 12 autosomal short tandem repeat (STR) loci in reference wolves (*Canis lupus*), wolves in the study area, reference dogs, and dogs sampled in the study area. H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; F_{IS} = inbreeding coefficient; P = probability to obtain F_{IS}-values higher than observed after 10,000 random permutations of alleles in each population computed by Genetix; N_A = average observed number of alleles per locus; N_E = expected number of alleles per locus (*SD* in parentheses).

Group	H_O	H_E	PIC	F_{IS}	P	N_A	N_E
Reference wolves	0.55 (0.21)	0.58 (0.22)	0.53 (0.20)	0.052 (0.057)	< 0.001	4.50 (2.78)	2.80 (1.06)
Wolves in the study area	0.56 (0.21)	0.57 (0.21)	0.52 (0.20)	0.037 (0.090)	0.350	5.25 (2.30)	2.69 (1.03)
Reference dogs	0.59 (0.12)	0.70 (0.13)	0.67 (0.13)	0.168 (0.081)	< 0.001	9.17 (3.49)	3.93 (1.80)
Dogs in the study area	0.58 (0.17)	0.68 (0.15)	0.64 (0.16)	0.152 (0.162)	< 0.001	8.17 (3.90)	3.98 (2.37)

frequent dog haplotypes, L and D, showed similar frequencies in reference (L = 35%; D = 26%) and noninvasively sampled dogs (L = 38%; D = 31%), but occurred at low frequency in the study area (L = 6%; D = absent) and in reference (L = absent; D = 3%; Table 1) wolves. All the 16 admixed genotypes (5 females and 11 males) showed the Italian wolf W14 mtDNA control-region haplotype. Six males shared the most frequent Italian wolf haplotype U, but the other 5 showed haplotypes 1, P, and O, which were found either in dogs or in non-Italian wolf populations (Supporting Information S6, DOI: 10.1644/13-MAMM-A-039.S6; Sundqvist et al. 2001; Caniglia et al. 2010; Iacolina et al. 2010).

Identification and composition of the wolf packs.—Wolves and hybrids were sampled from 1 to 56 times (Fig. 2A). Each genotype was sampled 4.7 times, on average, but 40% of the genotypes were sampled only once. The average sampling period per genotype was 12.6 months, and 21% of the genotypes were sampled for more than 24 months, up to more than 7 years (Fig. 2B). We identified 90 wolves (46 males and 44 females) that were sampled at least 4 times and for more than 24 months in areas smaller than 100 km^2. Their 95% kernel spatial distributions were partially overlapping and led to delimit 42 distinct areas, each of them including at least 1 frequently sampled male and 1 frequently sampled female (Table 3; Fig. 3). We sampled 280 other individuals within these areas and their surroundings of approximately 17 km (see "Results: *Spatial analyses and dispersal*") and, thus, we used 370 individuals to reconstruct the family groups in Colony. We identified pairs of genotypes in 26 of these areas, having probability $P > 0.90$ to be parents of 1 or more offspring, and reconstructed their familial pedigrees (Fig. 4). The pack pedigrees and locations suggested that the territories of the familial groups were roughly stable in time, but their compositions varied. In fact, we identified 34 putative breeding pairs in the 26 areas, corresponding to 63 reproductive wolves (32 males and 31 females). Parent–offspring genealogies were reconstructed for a total of 76 pack-

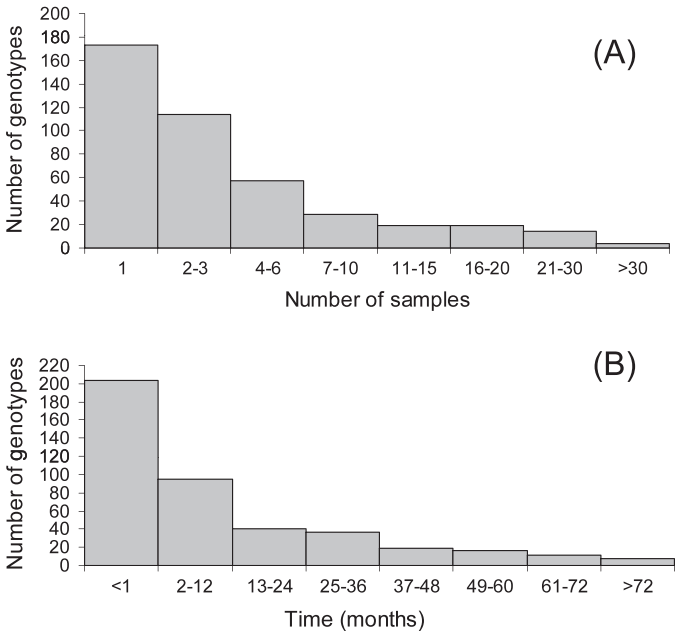

Fig. 2.—A) Number of samples per genotype. Individual resampling ranges from 1 to 56 (average = 4.7 ± 6.6 *SD*). B) Genotype sampling time (in months) from the 1st to the last sampling event (average = 12.6 ± 18.5 *SD*).

Table 3.—Wolf (*Canis lupus*) packs identified in this study. Pack areas were quantified by 95% fixed-kernel analysis using the least-square cross-validation method to choose band width (Seaman et al. 1999). Numbers indicate packs with reconstructed genealogies; letters indicate packs in which genealogies were not identified. ID = identification.

Pack name	ID	Area (km^2)	Pack name	ID	Area (km^2)
La Verna	1	52.78	Orecchiella	22	53.96
Badia Prataglia	2	20.37	Ligonchio	23	48.94
Sasso Fratino	3	52.20	Cerreto	24	89.77
Camaldoli	4	50.92	Ramiseto	25	144.43
San Paolo	5	45.57	Berceto	26	38.63
Falterona	6	99.26	Montironi	A	42.42
Castel dell'Alpe	7	79.96	Valpiana	B	57.74
San Benedetto	8	56.37	Sintria	C	50.05
Marradi	9	218.78	Sillaro	D	45.13
Castel del Rio	10	36.42	Casoni	E	243.63
Savena	11	67.33	Vaglia	F	50.27
Gessi	12	36.70	Loiano	G	46.27
Paderno	13	39.23	Casio	H	39.38
Monte Sole	14	56.67	Pavullo	I	137.10
Monte Vigese	15	29.88	San Lorenzo	J	74.33
Brasimone	16	54.29	Fiumalbo	K	45.78
Gaggio	17	59.45	Busana	L	107.55
Corno alle Scale	18	40.60	Carrega	M	86.45
Sestola	19	64.37	Corniglio	N	145.92
Pievepelago	20	102.80	Borgotaro	O	66.65
Frassinoro	21	32.00	Trebbia	P	211.96

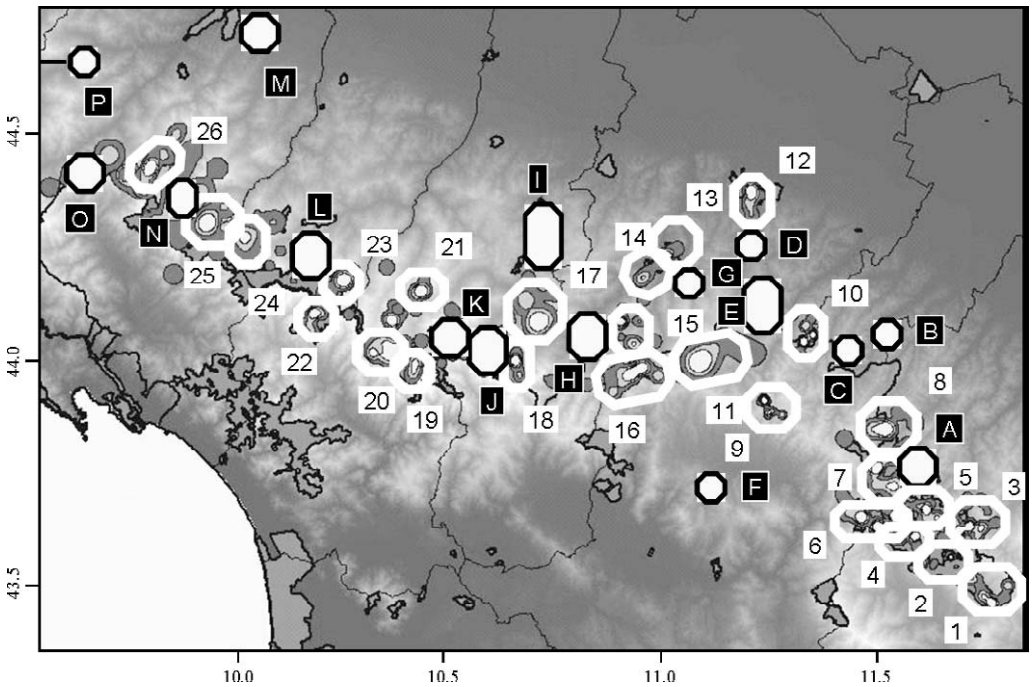

Fig. 3.—Fixed-kernel distribution (95% least-square cross-validation—Seaman et al. 1999) of the sampled wolf (*Canis lupus*) genotypes, with the approximate distribution of the 42 packs detected in the study area. White polygons (and numbers) indicate wolf packs with genealogies; black polygons (and letters) indicate wolf packs without genealogies. Longitude and latitude are indicated on the x- and y-axes in decimal degrees (datum WGS84).

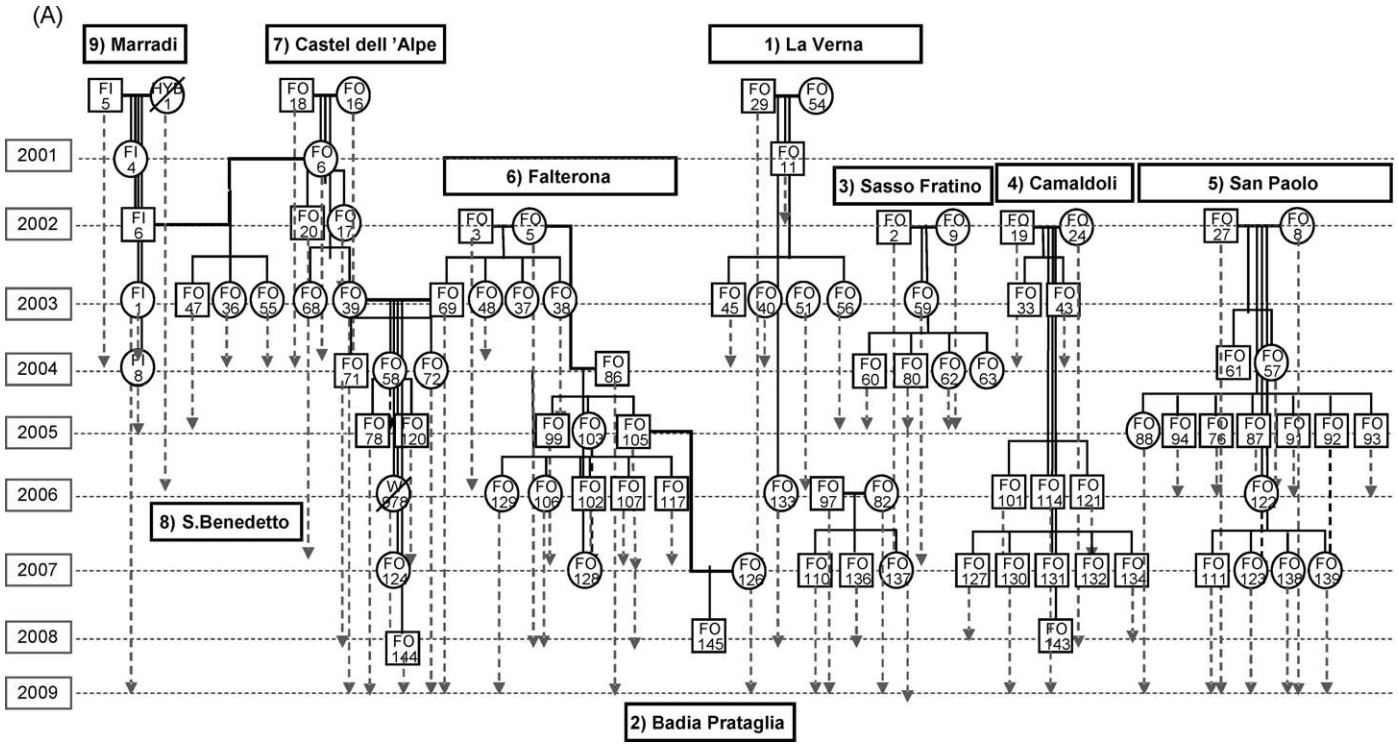

Fig. 4.—Genealogy of the 26 wolf (*Canis lupus*) packs identified in the study area (see: Tables 3 and 4; Supporting Information S5). Squares = males; circles = females. Thick continuous lines connect reproductive pair members; thin continuous line connect offspring groups. Vertical dashed arrows indicate the sampling period of each genotype. Slashes indicate found-dead wolves.

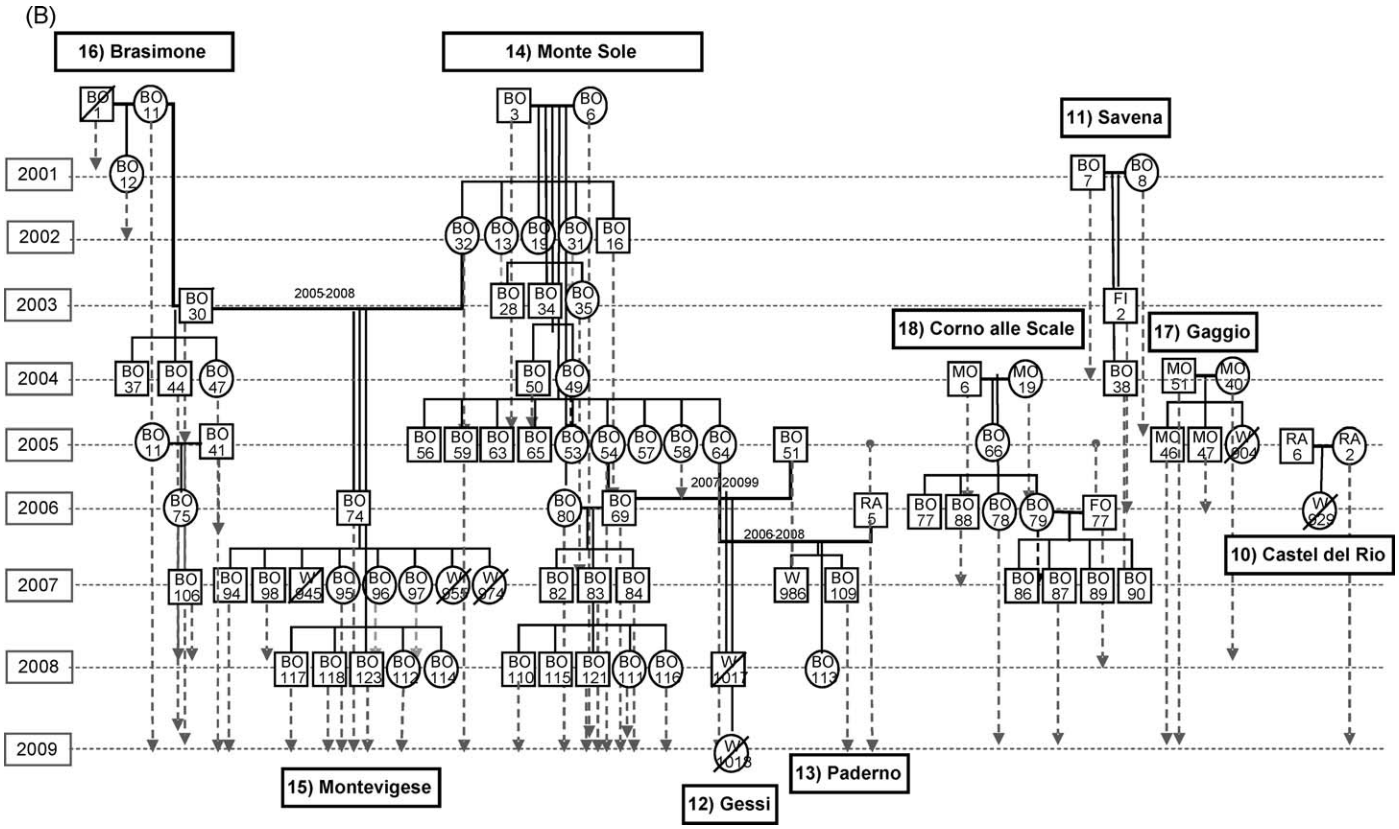

FIG. 4.—Continued.

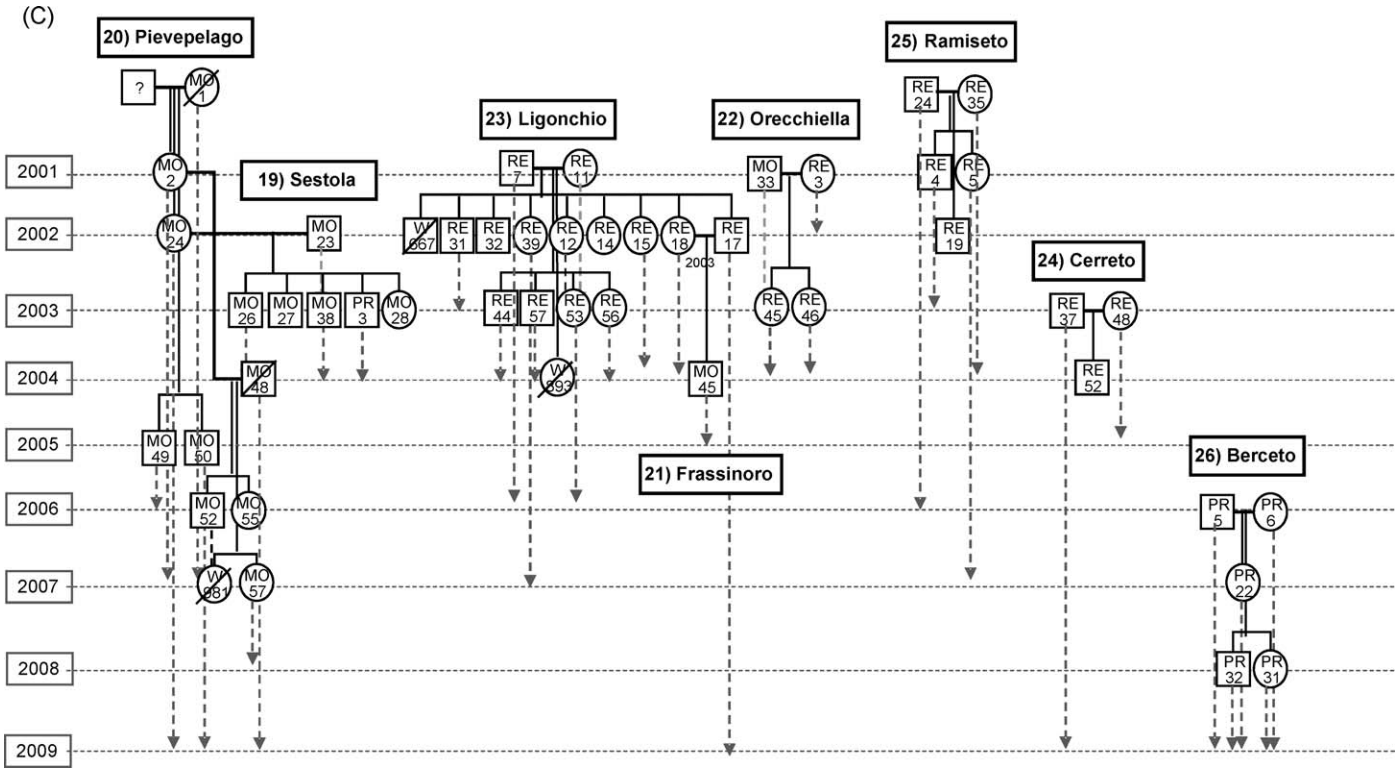

FIG. 4.—Continued.

years, including 4 cases where a single parent was identified (Fig. 4; Supporting Information S7A, DOI: 10.1644/13-MAMM-A-039.S7). The "open parentage analysis" in COLONY found 6 additional complete and 21 partial genealogies (9 father–offspring and 12 mother–offspring groups). These received much lower parent probabilities ($P < 0.50$), however, showed allelic incompatibilities and included individuals that were never contemporarily sampled in the same area but that were identified in other areas associated with unrelated wolves. CERVUS identified 198 clusters (1 parent pair plus several offspring) and 342 trios (parent pair plus only 1 offspring): 29 clusters (15%) and 114 trios (33%) corresponded to the 26 genealogies identified by COLONY. None of the alternative genealogies was supported by sampling dates, frequency, or location. Moreover, there were from 2 to 5 of 24 allele incompatibilities and incongruities at Y-haplotypes in 93 (95%) of 98 father–son combinations in the trios with significant natural log of likelihood ratio scores (Supporting Information S7B).

Packs included their breeding pairs and up to 11 related members, including the offspring of the year and yearlings (offspring of previous reproductions, see pack 14 in 2005 [Supporting Information S7A]), plus up to 3 unrelated individuals (in pack 16 in 2004 [Supporting Information S7A]). The average annual pack size including adoptees was 5.6 ± 2.4 individuals. The mean number of pups per pack (estimated by the number of pups sampled in late autumn) was 2.4 ± 2.0; the average number of yearlings per pack was 0.8 ± 1.0. Ten yearlings remained in their natal packs for more than 2 consecutive years (in packs 5, 6, 7, 14, 16, and 20). The mean number of unrelated individuals, sampled for at least 6 months in the packs' range, was 0.4 ± 0.7, and 4 of them remained in the same pack for 3 consecutive years (in packs 6, 16, and 25 [Supporting Information S7A]). The remaining 60 individuals (14% of the population) were never detected within or nearby the 42 pack areas. These represented potential floaters, which were sampled for an average of 5 ± 11 months, up to 6 years. Those included 15 of the 16 hybrids, because only 1 (HY1F) was identified as a member of a known pack (Supporting Information S6 and S7).

Relatedness and inbreeding.—A matrix of pairwise relatedness among all the individuals included in the packs showed that 33 (94%) of the 34 breeding pairs were significantly unrelated ($P < 0.05$; likelihood ratio test). Parents RE17M and RE18F in pack 21 showed a significant 1st-order relationship (brother and sister; $P = 0.011$), also confirming the genealogy of their natal pack, 23 (Fig. 4; Supporting Information S7B). The mean inbreeding coefficient of the breeding pairs was $F = –0.02$ (–0.25–0.30, 95% *CI*) ranging from –0.15 (–0.40–0.25) in pack 4 to 0.35 (0.09–0.73) in pack 7 (Table 4; Supporting Information S7B). The observed heterozygosity did not differ significantly between breeding pairs ($H_O = 0.57 \pm 0.15$, $n = 34$) and their offspring ($H_O = 0.54 \pm 0.22$, $n = 179$; $t_{33} = 1.52$, $P = 0.67$; *t*-test), or between breeders ($H_O = 0.57 \pm 0.48$, $n = 63$) and nonbreeders ($H_O = 0.56 \pm 1.15$, $n = 367$; $t_{62} = 2.35$, $P = 0.93$; *t*-test; Table 4).

The simulated distributions of pairwise relatedness values between unrelated (mean $r = –0.006 \pm 0.214$) and 1st-order wolves (mean $r = 0.487 \pm 0.164$) were partially overlapping (Fig. 5A). Following Lucchini et al. (2002), we fixed the limit for the individual classification at $r = 0.240$ (the midpoint between the averages of the 2 distributions), finding that 14% unrelated, 11% full-siblings, but only 4% parent–offspring pairs would be misclassified. The average relatedness estimated in the 26 wolf packs with pedigrees ($r = 0.390 \pm 0.106$) was significantly higher than in the whole population ($r = –0.014 \pm 0.289$; $t_{25} = 63.33$, $P < 0.0001$; *t*-test) and also higher than the fixed midpoint ($r = 0.240$; $t_{25} = 21.48$, $P < 0.0001$; *t*-test). Values of relatedness within wolf packs were variable, ranging from $r = 0.240 \pm 0.181$ (in pack 1) to $r = 0.682 \pm 0.271$ (in pack 11 [Supporting Information S7B]).

Spatial analyses and dispersal.—The 95% kernel analysis showed that the packs were settled in a minimum total area of 3,122 km^2 (one-sixth of the sampling area of 19,171 km^2 [Fig. 3]). The average 95% kernel area of individuals belonging to packs was 35.72 ± 20.20 km^2 (ranging from 4.51 to 170.64 km^2); the average pack area was 74.34 ± 51.69 km^2 (ranging from 20.37 to 243.63 km^2; 42 packs) or 60.02 ± 41.39 km^2 (ranging from 20.37 to 218.62 km^2) when computed for the 26 areas with reconstructed genealogies (Table 3). The autocorrelation of kinship versus the logarithmic interindividual distance was significantly negative ($b = –0.013 \pm 0.010$; $P < 0.001$). Positive values of the F_{ij} kinship coefficient at short distances indicated that geographically closer wolves had higher-than-expected kinship, whereas negative values at long distances highlighted isolation by distance (Fig. 5B). The x-intercept on the autocorrelogram suggested that within 17 km wolves are more closely related to one another than on average across the population. Thus, we considered as potential dispersers 27 wolves that were successively sampled in different locations farther than 17 km, and 10 wolves that stably settled in a pack different from their original one, but at shorter distances (Table 5). Twenty wolves (54%) dispersed southeast to northwest, toward the Alps. The average dispersal distance was 52.97 ± 40.17 km. Dispersal was significantly male-biased (26 individuals, $\chi^2_1 = 6.06$, $P < 0.01$; chi-square test), as suggested also by autocorrelation analyses in male and female distance classes, which showed higher relatedness among females ($r = 0.090$; 11 km) than males ($r = 0.070$; 20 km). Twenty-two (59%) of the 37 dispersers apparently settled in a new pack, and 14 (38%) of them also became breeders: 2 males established and reproduced in already existing packs, and 12 (5 males and 7 females) founded their own new packs. In comparison, only 5 (26%) of 19 known nondispersing individuals (wolves born and sampled in the same pack for at least 3 years) became breeders in their natal pack (4 females and 1 male). Another 15 dispersers (13 males and 2 females) were never detected in association with a known pack (2), or were born in a known pack (13) but dispersed to unoccupied areas, thus representing other potential cases of floaters.

Table 4.—Pack number, name, and composition of wolves (*Canis lupus*); identification of the breeding males (BM) and females (BF); total number of offspring per pair (N_O); estimates of relatedness ± *SD* (*r*—Queller and Goodnight 1989) and inbreeding coefficient (*F*; 95% confidence intervals in parentheses—Lynch and Ritland 1999) between parents; and estimates of observed heterozygosity (H_O ± *SD*) in parents and offspring.

Pack	Name	BM[a]	BF[a]	N_O	$r_{parents}$	$F_{parents}$	$H_{O\ parents}$	$H_{O\ offspring}$
1	La Verna	FO29M	FO54F	6	−0.03 ± 0.03	−0.03 (0.24–0.22)	0.58 ± 0.11	0.60 ± 0.24
		FO97M	FO82F	3	−0.29 ± 0.03	0.09 (−0.12–0.49)	0.50 ± 0.13	0.67 ± 0.16
2	Badia Prataglia	FO105M	FO126F	1	0.04 ± 0.10	0.10 (−0.22–0.65)	0.46 ± 0.14	0.33 ± 0.14
3	Sasso Fratino	FO2M	FO9F	5	−0.06 ± 0.08	−0.09 (−0.31–0.65)	0.67 ± 0.13	0.55 ± 0.20
4	Camaldoli	FO19M	FO24F	2	0.01 ± 0.04	−0.09 (−0.33–0.22)	0.67 ± 0.13	0.42 ± 0.17
		FO34M	FO24F	9	−0.18 ± 0.03	−0.15 (−0.40–0.25)	0.63 ± 0.13	0.62 ± 0.24
5	San Paolo	FO27M	FO8F	14	0.07 ± 0.03	−0.11 (−0.37–0.20)	0.54 ± 0.18	0.52 ± 0.41
6	Falterona	FO3M	FO5F	6	−0.04 ± 0.02	−0.08 (−0.25–0.23)	0.58 ± 0.17	0.49 ± 0.27
		FO86M	FO5F	7	0.45 ± 0.02	−0.11 (−0.34–0.03)	0.71 ± 0.16	0.48 ± 0.21
7	Castel dell'Alpe	FO18M	FO16F	5	−0.05 ± 0.07	0.10 (−0.08–0.54)	0.46 ± 0.16	0.63 ± 0.25
		FO69M	FO39F	8	−0.14 ± 0.01	0.35 (−0.09–0.73)	0.38 ± 0.13	0.58 ± 0.28
8	San Benedetto	FI6M	FO6F	3	0.15 ± 0.04	0.13 (−0.17–0.44)	0.58 ± 0.11	0.69 ± 0.13
9	Marradi	FI5M	HYB1F	4	−0.29 ± 0.01	−0.09 (−0.24–0.29)	0.58 ± 0.17	0.73 ± 0.22
10	Castel del Rio	RA6M	RA2F	1	−0.03 ± 0.05	0.06 (−0.23–0.40)	0.54 ± 0.16	0.50 ± 0.15
11	Savena	BO7M	BO8F	2	0.43 ± 0.05	0.09 (−0.25–0.57)	0.46 ± 0.16	0.42 ± 0.20
12	Gessi	BO51M	BO54F	2	−0.25 ± 0.05	−0.03 (−0.28–0.22)	0.58 ± 0.14	0.58 ± 0.11
13	Paderno	RA5M	BO64F	3	−0.16 ± 0.02	−0.05 (−0.18–0.11)	0.63 ± 0.16	0.44 ± 0.16
14	Monte Sole	BO3M	BO6F	20	−0.33 ± 0.03	−0.09 (−0.27–0.05)	0.67 ± 0.13	0.64 ± 0.40
		BO69M	BO80F	8	0.24 ± 0.02	−0.04 (−0.25–0.25)	0.58 ± 0.20	0.47 ± 0.34
15	Monte Vigese	BO30M	BO32F	14	−0.22 ± 0.04	−0.10(−0.33–0.03)	0.75 ± 0.17	0.62 ± 0.37
16	Brasimone	BO1M	BO11F	1	0.69 ± 0.02	0.00 (−0.23–0.50)	0.46 ± 0.18	0.17 ± 0.11
		BO30M	BO11F	3	−0.13 ± 0.04	−0.08 (−0.35–0.25)	0.63 ± 0.16	0.56 ± 0.24
		BO41M	BO11F	2	−0.20 ± 0.03	−0.11 (−0.36–0.26)	0.63 ± 0.13	0.58 ± 0.14
17	Gaggio	MO51M	MO40F	3	0.20 ± 0.03	−0.06 (−0.24–0.28)	0.50 ± 0.16	0.39 ± 0.16
18	Corno Scale	MO6M	MO19F	5	−0.07 ± 0.10	0.07 (−0.26–0.41)	0.58 ± 0.11	0.63 ± 0.22
		FO77M	BO79F	4	−0.16 ± 0.03	−0.02 (−0.28–0.22)	0.63 ± 0.18	0.67 ± 0.20
19	Sestola	MO23M	MO24F	5	0.04 ± 0.02	−0.13 (−0.29–0.14)	0.71 ± 0.14	0.58 ± 0.20
20	Pievepelago	MO48M	MO2F	4	−0.08 ± 0.03	−0.11 (−0.24–0.14)	0.58 ± 0.20	0.58 ± 0.24
21	Frassinoro	RE17M	RE18F	1	0.45 ± 0.02	−0.04 (−0.34–0.42)	0.58 ± 0.17	0.50 ± 0.15
22	Orecchiella	MO33M	RE3F	2	0.37 ± 0.03	0.02 (−0.18–0.47)	0.42 ± 0.17	0.54 ± 0.20
23	Ligonchio	RE11M	RE7F	14	−0.05 ± 0.02	−0.01 (−0.28–0.39)	0.54 ± 0.10	0.55 ± 0.30
24	Cerreto	RE48M	RE37F	1	0.06 ± 0.09	0.12 (−0.31–0.39)	0.54 ± 0.14	0.58 ± 0.15
25	Ramiseto	RE35M	RE24F	3	−0.24 ± 0.06	−0.12 (−0.23–0.12)	0.63 ± 0.16	0.61 ± 0.19
26	Berceto	PR5M	PR6F	3	−0.07 ± 0.08	−0.06 (−0.28–0.13)	0.54 ± 0.14	0.54 ± 0.21
Average				5.1 ± 4.5	0.004 ± 0.040	−0.02 (−0.25–0.30)	0.57 ± 0.15	0.54 ± 0.22

[a] In the individual identificationss, the first 2 letters indicate the province where the individual was 1st sampled (see Fig. 1), the number is a unique identifier within each province, and the last letter indicates the sex (M = male; F = female).

Wolves dispersing short distances (19.2 km, on average) apparently had higher likelihood ($F_1 = 27.71$, $P < 0.0001$; 1-way analysis of variance) to reproduce in new packs than wolves moving longer distance (73.5 km, on average). The founders were born in areas close to the centroids of the new packs, mapping at an average distance of 17.5 ± 12.2 km, thus explaining why the observed isolation by distance and autocorrelations dropped at a short geographical distance.

Pack member dynamics.—Pack composition and dynamics are summarized in Table 6. In the eastern sector of the study area (lower-right side of Fig. 3), we reconstructed the complete genealogies in 9 of the 12 documented packs (Fig. 4A). The breeding pairs in 6 packs were stable throughout the study (packs 2, 3, 4, 5, 8, and 9, detected from 1 to 6 years; average 3.5 years [Supporting Information S7A]). In pack 1 (La Verna), both parents detected since 2001 were completely replaced in 2006 by a new breeding pair of unknown familial origin that reproduced in 2007. Other packs showed more-complex dynamics. Offspring from packs 9 (Marradi) and 7 (Castel dell'Alpe) joined in a breeding pair that in 2002 founded a new pack in between the 2 packs (8, San Benedetto). Pack 7 showed a turnover of the reproductive pair identified in 2001, which apparently disappeared in 2003, when it was replaced by a female offspring born in 2002 and by an unrelated immigrant male born in 2002 in pack 6 (Falterona). The reproductive male in pack 6 was replaced in 2004 by an immigrant male of unknown familial origin. A male offspring of the new Falterona pack established a new pack in 2007 in an adjacent area (2, Badia Prataglia), mating with an unrelated female of unknown familial origin. The number of detected packs increased from 7 (in 2001–2002) to 9 (in 2008–2009). Both the new packs filled new areas. Three (43%) of the original 7 packs were genetically connected through replacements (1) or new pack foundations (2). The presence of 6 of the 7 packs identified in 2001 in this sector was confirmed by wolf-howling

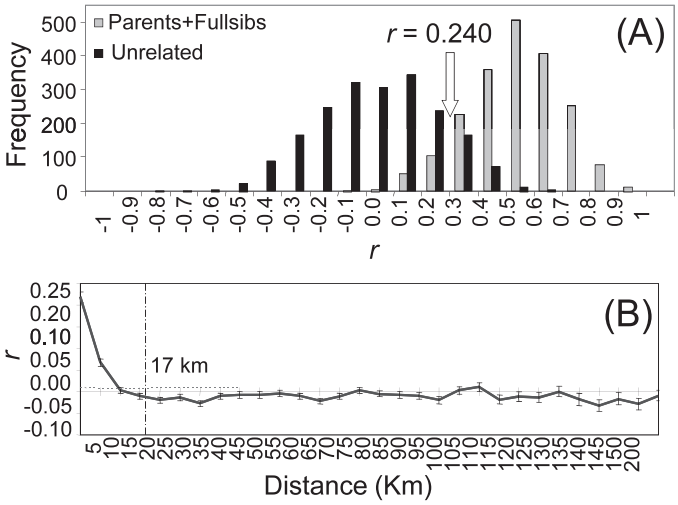

Fig. 5.—A) Distributions of relatedness (*r*) of Queller and Goodnight (1989) for 1st-order (parents–offspring plus full siblings) relatives and unrelated individuals obtained from 1,000 dyads simulated in KinGroup (Konovalov et al. 2004) using allele frequencies from the wolf (*Canis lupus*) population. The arrow indicates the midvalue between the 2 distributions. B) Autocorrelogram of relatedness (*r*) of Queller and Goodnight (1989) against distance class sizes of 5 km in wolves (95% confidence interval values for *r* were calculated for each distance class by bootstrap).

sessions (Caniglia et al. 2010), which also confirmed the presence of 8 packs in 2006 (Supporting Information S7A).

In the central part of the study area (Fig. 3), we reconstructed complete genealogies in 9 (packs 10–18) of the 15 mapped packs (Fig. 4B). In 6 packs (10, 11, 12, 13, 15, and 17) the breeding pair did not change during the study. In the other 2 packs (14, Monte Sole; 18, Corno alle Scale), on the contrary, the breeding pairs were completely replaced by female offspring and immigrant males of unknown familial origin. In pack 16 (Brasimone) female BO11F reproduced from 2001 to 2008, but the breeding males changed 3 times (in 2003, 2004, and 2006). One of them, BO30M, later colonized an adjacent territory and established a new pack (15, Monte Vigese) with a female born in 2002 in pack 14 (Monte Sole). Two females from pack 14 originated 2 new groups, pack 12 (Gessi) in 2007, and 13 (Paderno) in 2008, with 2 males of unknown familial origin. One of them, male RA5M, was sampled 2 years before about 50 km away in a straight line, similar to male FO77M, which replaced the previous breeder in pack 18 (Corno alle Scale). Thus, in this central region of the study area, pack interchanges involved 3 (33%) of 9 genealogies. Each time replacements involved the immigration of unrelated males that mated with resident females. Three new packs (12, 13, and 15) originated from unrelated individuals migrating from neighboring zones and filling new areas. Packs increased from 6 (in 2001–2004) to 8 (in 2008), although it was not possible to reconstruct all their genealogies. Whenever carried out, field surveys confirmed the results from genetic data (Supporting Information S7A): the minimum number of wolves was confirmed by snow-tracking in packs 10, 11, 14, 16, and 18 (Caniglia et al. 2010); wolf-howling detected reproductions in packs 10, 11, 12, 14, 15, 16, and 18 (Caniglia et al. 2010); and camera trapping confirmed 2 reproductions and the minimum number of wolves in pack 18 (Galaverni et al. 2012).

Table 5.—Identification of wolves (*Canis lupus*) that likely dispersed from their natal packs. Genotype identification (ID), sex, dispersal direction, distance from the putative natal area (km), and minimum permanence (in years) in the new areas are shown (whenever known, pack ID numbers are indicated [see Tables 3 and 4; Fig. 4]); evidence of reproduction (R) in the destination pack and of being the founder of a new pack (F) are indicated.

Genotype ID	Sex	Direction	km	Permanence (years)	Pack of destination
BO88M	M	SE–NW	150	< 1	
FO15M	M	SE–NW	142	4	
FO46F	F	SE–NW	124	2	
BO10M	M	SE–NW	114	< 1	
BO44M	M	SE–NW	105	2	
BO87M	M	SE–NW	105	< 1	
FI12M	M	SE–NW	85	< 1	
RE23M	M	NW–SE	80	5	
RE39F	F	NW–SE	77	< 1	
RE6M	M	NW–SE	76	4	
FO77M	M	SE–NW	67	3	17 (R)
BO16M	M	SE–NW	66	3	
FO61M	M	SE–NW	66	3	
RE51F	F	SE–NW	65	< 1	
PR15M	M	SE–NW	65	< 1	
MO46M	M	SE–NW	64	2	
PR3M	M	SE–NW	53	1	
FO25M	M	SE–NW	52	5	
RA5M	M	SE–NW	50	1	12 (R, F)
PR4F	F	SW–NE	49	< 1	
FO92M	M	NE–SW	43	< 1	
BO38M	M	SE–NW	34	3	
RE4M	M	NW–SE	27	1	
BO54F	F	SW–NE	27	1	11 (R, F)
FO47M	M	SE–NW	25	2	
FO130M	M	NE–SW	24	< 1	
BO6F	F	SW–NE	21	3	13 (R, F)
BO64F	F	SW–NE	17	1	12 (R, F)
FI6M	M	SE–NW	15	3	8 (R, F)
MO24F	F	NW–SE	13	< 1	18 (R, F)
RE17M	M	NW–SE	12	4	20 (R, F)
RE18F	F	NW–SE	12	2	20 (R, F)
FO105M	M	NE–SW	10	2	2 (R, F)
BO30M	M	SW–NE	8	3	14 (R, F)
BO32F	F	NE–SW	7	6	14 (R, F)
FO69M	M	SW–NE	5	4	7 (R, F)
FO6F	F	SE–NW	5	< 1	8 (R, F)

Eight pedigrees (Fig. 4C) were reconstructed in the 15 packs identified in the western sector of the study area (upper-left part of Fig. 3). In pack 20 (Pievepelago), a female offspring colonized an adjacent territory and founded a new pack (19, Sestola) with a male of unknown genealogy, whereas her sister replaced their mother as a breeder in 2004. A case of incestuous mating was detected in 2003: a brother and a sister from pack 23 (Ligonchio) mated and originated the new pack 21 (Frassinoro). The other 4 packs were apparently not

Table 6.—Wolf (*Canis lupus*) pack number, composition, and dynamics in the study area. The table indicates number of packs that set up and stably use their own territorial areas; number of packs with reconstructed genealogies; average pack size (including unrelated individuals sampled in the pack range); average pack size including only related individuals as inferred from the genealogies; and sex ratio computed only among related wolves. Pack dynamics indicates changes due to complete or partial replacements of breeders by unrelated or immigrant wolves, or by offspring of the previous breeding pairs. The number of new packs, founded by unrelated or related wolves, also is indicated (U = documented usurpation: an immigrant usurps an active breeder that was still sampled in the pack area; KP = an immigrant from a known pack replaces the breeder; UP = an unrelated or immigrant wolf from an unknown pack or area replaces the breeder).

	n	No. males	No. females
Pack number and composition			
Packs in the study area	42	187	144
Packs with genealogy	26	138	108
Average pack size	5.55		
Average pack size/year (only related wolves)	5.13	2.55	2.59
Sex ratio/year (only related wolves)	1.25		
Pack dynamics			
Complete replacements within packs	5		
By 2 new immigrant, unrelated wolves	1 (1U male)	1 (1UP)	1 (1UP)
By 1 immigrant, unrelated male and 1 female offspring of the previous pair	4 (3U females, 1U both male and female)	4 (3UP, 1KP)	4 (KP)
By 2 offspring of the previous pair	0		
Partial replacements within packs	3		
By 1 offspring of the previous pair	0		
By 1 immigrant, unrelated wolf	3 (3U male)	3 (3UP)	
New packs founded by 2 dispersing individuals	7		
By 2 unrelated individuals	6	6 (4KP, 2UP)	6 (5KP, 1UP)
By 2 related individuals (brother and sister)	1	1 (1KP)	1 (1KP)

interconnected by any exchange of immigrant or dispersal individuals. The number of packs increased from 6 (in 2001–2003) to 8 (in 2008), although it was not possible to reconstruct all their genealogies, and the 2 new packs filled vacant areas. Four packs were confirmed (Supporting Information S7A) by wolf-howling in 2002 (packs 19, 20, 22, and 24), and 6 (19, 20, 22, 23, 24, and 25) by snow-tracking between 2002 and 2004 (Life Project 2004).

Discussion

Molecular identifications of DNA samples collected noninvasively over 9 years led us to obtain the most complete description to date of the distribution range and demographic structure of a wolf population living in a wide area of the Apennine Mountains. We identified 414 distinct wolf genotypes (plus 88 dogs and 16 wolf × dog hybrids) in a population that is estimated to average 187 ± 78 wolves (data extrapolated from results described by Caniglia et al. [2012]). We located 42 packs and fully reconstructed the genealogies of 26 of them. Through the identification of resident wolves in packs we inferred the number and destiny of dispersers and obtained a rough estimate of floaters. The wealth of information gathered by noninvasive genetic sampling projects of this kind could have not been obtained with any other monitoring tool at a comparable cost (Galaverni et al. 2012).

Our study relied on molecular identifications of samples that were collected year-round by trained collaborators. Although accurate selection of fresh scat samples was not guaranteed, genotyping success was comparable to values reported in other noninvasive genetic studies of carnivores (e.g., 14–63% in otters, 54% in wolverines, 48–61% in wolves, and 45% in pine martens [as reported by Ruiz-González et al. 2013]). Moreover, the absence of seasonal effects (see Supporting Information S2; Santini et al. 2007) indicates that large-scale surveys could focus either on winter (e.g., sampling on snow tracks—Lucchini et al. 2002) or summer sampling (e.g., at rendezvous sites—Stenglein et al. 2011). We realize that nonsystematic sampling procedures may miss portions of the target population, particularly in areas difficult to access or that were recently colonized. However, fully randomized sampling schemes will probably remain too expensive for monitoring elusive, low-density, and widespread large carnivores (Duchamp et al. 2012). Through nonsystematic, but protracted, noninvasive genetic sampling it is possible to reconstruct detailed wolf pack territory maps, which facilitate the identification of distribution gaps and the design of optimally allocated transects in predefined sampling grid cells. This approach is useful in monitoring demographic and genetic trends in wolves and other species of canids and elusive carnivores, also found in regions where dense forest cover or absence of snow periods prevent the use of field-monitoring methods (Blanco and Cortés 2012).

A main benefit of noninvasive genetics is the inference of wild pedigrees, reliability of which depends upon the proportion of sampled parents (which, in theory, should almost all be sampled), and the power of the genetic markers to exclude or assign each individual to a single parental class with high probability (Kalinowski et al. 2007; Pemberton 2008). In this study we did not obtain independent estimates of the

proportion of sampled parents, because no field method was practicable at such a large scale. However, simulations showed that the risk to misidentify parent–offspring dyads was small (4%), and kinship analyses consistently partitioned the samples into a set of well-supported trios or dyads (parent–offspring and full-siblings) versus a set of unsupported kinships. Pack identifications and their genealogies can be used as working hypotheses to provide real-time descriptions of wolf colonization patterns, eventually indicating obstacles to dispersal and local patches of inbreeding or hybridization, which should be quickly managed by appropriate conservation actions.

Genetic variability and the assessment of wolf × dogs hybridization.—The continuing wolf expansion in human-dominated landscapes, where free-ranging dogs are frequent and disturbance is heavy, increases hybridization risks. The 16 hybrids found in the study area correspond to approximately 4% of the sampled individuals. Similar frequencies were reported in Iberia (Godinho et al. 2011), the Baltic countries (Hindrikson et al. 2012), and wolves randomly collected from the entire distribution range in Italy (Verardi et al. 2006). Despite hybridization, all the studied wolf populations in Europe remain genetically distinct from dogs (Verardi et al. 2006; Godinho et al. 2011; Hindrikson et al. 2012), suggesting that backcrossing in wolves is not frequent or it is constrained by natural selection (Randi 2011). However, most published wolf studies used fewer than 40–50 microsatellites and have limited power to identify hybrids beyond the first 2 or 3 generations of backcrossing in populations diverging at $F_{ST} <$ 0.10–0.15 (Vähä and Primmer 2006). Improved identifications of admixed genotypes will be obtained not simply by expanding the number of markers, which will be unsustainable in conservation projects, but by genotyping limited numbers of very informative mutations (Axelsson et al. 2013; vonHoldt et al. 2013). We anticipate that forthcoming conservation genomic approaches (Steiner et al. 2013) will provide more efficient tools for deeper assessments of hybridization (Rutledge et al. 2012).

Improved molecular identification methods and more comprehensive data sets, however, should be analyzed in proper logical frameworks. In this perspective straight assessments of hybrid frequency should be integrated with genealogical reconstructions to identify the number and locations of the original hybrid packs that contribute to the diffusion of hybrid individuals. We hypothesize that, because of Allee effects and their genetic consequences (Roques et al. 2012), wolf × dog hybridization is more frequent at the edges of expanding populations (see also Godinho et al. 2011). Large-scale, noninvasive genetic monitoring of expanding populations will help to test this prediction, and will contribute to designing efficient plans to contrast hybridization. Spatial and temporal dynamics of hybridization and backcrossing in wolves and other canids are conditioned by landscape features and anthropogenic factors (Benson and Patterson 2013). Georeferenced genotype data and habitat variables could be modelled, reconstructing maps of hybridization risk, thus providing important resources for the monitoring and management of hybridizing canid populations.

Pack size and composition.—Wolf pack territories are regionally variable and reflect latitudinal clines or variation in prey density and composition (Fuller et al. 2003; Ciucci et al. 2009). The ranges of the 42 pack territories in the study area (74 ± 52 km^2) as estimated by noninvasive genetics was smaller than in other wolf populations in Europe (80–300 km^2—Kusak et al. 2005; Jędrzejewski et al. 2007) and North America (100–800 km^2— Mech 1999; Fuller et al. 2003), but similar to estimates from previous studies in comparable ecological contexts in Italy (approximately 50–200 km^2—Apollonio et al. 2004; Scandura et al. 2011). As hypothesized, neighboring packs in the Apennine Mountains have mostly nonoverlapping territories. The observed interpack distance (8–16 km) compares well to the limit of nonrandom genetic structure estimated by autocorrelation analyses (17 km) as well as with results from other studies (Apollonio et al. 2004; Scandura et al. 2011). The spatial distributions of noninvasive samples are conditioned by sample collection and certainly biased the estimate of pack territories, which are probably closer to pack core areas than to their wider home ranges. Large-scale, noninvasive genetic monitoring offers preliminary, perhaps coarse, estimates of pack territory sizes and shapes that could be determined with more details by global positioning system or radiotracking studies, which, however, remain difficult and expensive in widespread populations of wolves and other canids. The reconstruction of wolf core ranges through noninvasive genetic sampling indicated that pack locations are stable in time, although pack composition is variable because of high turnover rates of the parental pairs. Pack locations indicate territories that are highly suitable to sustain wolf presence and reproduction, and are useful to field biologists and managers to plan more efficient field monitoring (e.g., wolf-howling) and conservation activities (e.g., prevention of livestock depredations).

Estimating pack size is conditioned by methodological constraints and published data are extremely variable (Blanco and Cortés 2012). Pack size could evolve, at least in part, to maximize group hunting success, thus it should vary according to the composition of the main prey communities (MacNulty et al. 2009). The average pack size in our study (5.5 ± 2.4) falls within the range of wolves in Europe (Fuller et al. 2003; Mech and Boitani 2003; Nowak et al. 2008; Marucco et al. 2009), suggesting that genetic and field methods produce comparable results. Assuming that 70% of packs reproduce each year, on average (Hayes and Harestad 2000), the mean annual population in the study area would be approximately 162 wolves, plus approximately 14–17% of floaters (which represent a temporary status of individuals that may later immigrate and eventually mate into existing packs; e.g., wolf 302M [vonHoldt et al. 2008]). Our estimate of floaters is slightly higher than those reported in other studies (10–15%—Fuller et al. 2003), but can be biased because some packs and genealogies may have been missed because of insufficient sampling. Genetic estimates of pack size can be compared to

independent estimates obtained through implemented bioacoustic methods (Root-Gutteridge et al. 2013), and applied to presence–absence and capture–recapture surveys of wolf and other carnivore populations.

Pack dynamics and inbreeding.—Inbreeding may reduce adaptability and increase demographic stochasticity in cooperative-breeding species and in small isolated populations. A number of inbreeding-avoidance behaviors, including juvenile dispersal, hierarchical control of reproductions, extra-pair reproduction, and pack turnover have evolved in carnivores (vonHoldt et al. 2008). In our case study, the reconstruction of multigeneration pedigrees indicated that inbreeding was a rare exception: all mating events involved unrelated individuals, with the exception of 1 brother–sister pair that founded a new pack after a probable splitting. Pack turnover was high (27%), and new packs were founded by unrelated wolves. Replacers were mainly unrelated males (67%) that apparently replaced dead or not resampled wolves, mating with offspring females that replaced their mothers within the natal pack (50% of the cases). This mechanism guarantees the production of offspring unrelated to the previous males (Jędrzejewski et al. 2005) and at the same time maintains pack stability. We also observed cases of complete replacements of the breeding pairs by 2 unrelated immigrant wolves, and partial replacement of the male breeder by an immigrant, unrelated wolf. We never observed replacer immigrant females mating with the pack males, nor complete replacements of both breeders by 2 of their offspring (vonHoldt et al. 2008). We never detected multiple litters per year in a pack, or extra-pair reproductions, which may constitute exceptional events favored by extreme conditions of food availability or in highly exploited packs (vonHoldt et al. 2008; Stenglein et al. 2011), which is not the case in our population.

The frequency of these behavioral mechanisms is variable in the studied wolf populations (vonHoldt et al. 2008; Stenglein et al. 2011), but all concur to minimize inbreeding and its negative consequences on fitness. All studies published so far (Sillero-Zubiri et al. 1996; Randall et al. 2007; vonHoldt et al. 2008) indicate that juvenile dispersal, pairing, and pack turnover concur to favor gene flow among packs in canids. Furthermore, Geffen et al. (2011) and Sparkman et al. (2012) suggested that selection for inbreeding avoidance may be weak in canids, because the low probability of kin encounters is enough to prevent inbreeding. Wild genealogies, validated by genetic identifications at nonfunctional and putatively neutral markers (such as microsatellites), can be used to test for hypothetical inbreeding-avoidance mechanisms, for instance by typing functional genes involved in kin recognition, such as genes in the major histocompatibility complex (Aguilar et al. 2004) and the olfactory receptors (Quignon et al. 2012), thus opening new ways to behavioral genetic studies in wild populations.

Pack dynamics and gene flow.—Individual replacements and new pack foundations detected in our study area were due to short-distance migrants. We found that 38% of dispersers became breeders in new or in already existing packs, ensuring interpack connection and gene flow. This mechanism helps in maintaining high genetic connections among adjacent packs, reduces within-pack relatedness and indicates that short-term effective gene flow is limited to a few kilometers around the pack territory (Scandura et al. 2011). On the other hand, long-distance dispersal provides a faster way to colonize new suitable areas during the early phase of population expansion (Fabbri et al. 2007). In both cases, dispersal is mostly male-biased. The average observed heterozygosity was not significantly different between breeding pairs and their offspring, and between breeding and nonbreeding individuals, further excluding major intergeneration shifts toward more inbred or more heterozygous offspring cohorts (Bensch et al. 2006; vonHoldt et al. 2008). This diversity of mating schemes reflects the dynamic condition of the expanding wolf population in Italy. However, poaching and incidental killings, the major causes of wolf mortality in Italy (Ciucci et al. 2007), are among the main determinants of pack instability. Fourteen wolves found dead were assigned to known packs; 10 of them were found within 1 year from the presumed pack foundation and 4 were killed by poaching and car accidents. Although expanding wolf populations can sustain high levels of human-caused mortality (Stenglein et al. 2011), its reduction would help to maintain the social structure of the packs and ensure the long-term conservation of the population. Moreover, high mortality and pack disruption may increase the risk of hybridization with dogs in expanding canid populations, particularly at the edge of the expansion waves.

Temporal trends in abundance and density are key parameters for wildlife conservation, but they are challenging to obtain in widespread elusive species such as some carnivores (Boitani et al. 2012). Extensive noninvasive genetic sampling and molecular identifications, possibly integrated with field data, provide the kind of genetic and demographic information needed by conservation programs of wolves and other carnivores. Although our results may not be generalized to other populations, the empirical data obtained in this study can be used to perform demographic analyses (Caniglia et al. 2012) and monitor future demographic trends in the Apennine Mountain wolf population. Such a large genetic database also could be used for implementing maps of predation risk and predictive habitat models of wolf expansion, monitoring hybridization, and assisting in forensic investigations (Caniglia et al. 2013). The demography and population genetics of canids and other species of large or midsize carnivores could be studied by well-planned, long-term, noninvasive genetic monitoring.

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Supporting Information

Supporting Information S1.—Description and performance of the multiple-tube approach and postprocess quality controls: multitube workflow.

Found at DOI: 10.1644/13-MAMM-A-039.S1

Supporting Information S2.—Description and performance of the multiple-tube approach and postprocess quality controls: laboratory methods.

Found at DOI: 10.1644/13-MAMM-A-039.S2

Supporting Information S3.—Description and performance of the multiple-tube approach and postprocess quality controls: genotyping steps and success.

Found at DOI: 10.1644/13-MAMM-A-039.S3

Supporting Information S4.—Description and performance of the multiple-tube approach and postprocess quality controls: results of mismatch analysis.

Found at DOI: 10.1644/13-MAMM-A-039.S4

Supporting Information S5.—Genetic variability by locus and population.

Found at DOI: 10.1644/13-MAMM-A-039.S5

Supporting Information S6.—List of the detected wolf × dog hybrids.

Found at DOI: 10.1644/13-MAMM-A-039.S6

Supporting Information S7.—Pack composition and dynamics.

Found at DOI: 10.1644/13-MAMM-A-039.S7

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