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ORIGINAL PAPER

Monitoring wolves (*Canis lupus*) by non-invasive genetics and camera trapping: a small-scale pilot study

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Abstract Monitoring populations of elusive large carnivores like wolves (*Canis lupus*), which are often distributed at low density in widespread forested areas, is difficult or exceedingly expensive. Aiming to assess the power of two indirect monitoring methods, non-invasive genetic sampling and camera trapping, we designed a small-scale pilot study that was carried out from 2006 to 2008 in and around the Corno alle Scale Regional Park, Bologna, northern Italian Apennine. We collected 103 non-invasive samples (mainly scats) that were genotyped at 12 microsatellite loci and sexed using the ZFX gene. We identified 11 distinct wolf genotypes within the park and four wolf genotypes outside. Spatial locations and kinship analyses showed that the wolves belong to three different packs. The breeding pair of the 'Park' pack showed a complete turnover in the two sampling seasons. Two dogs, but no hybrids, were identified in the area. Up to five unbaited camera traps were activated (for 1,250 trapping-nights) close to recent wolf presence marks. We obtained 103 photos of wolves, documenting the reproduction events, the minimum number of adult and young wolves, and phenotype information each year. We obtained information on health conditions detecting probable sarcoptic mange in three individuals. Camera trapping also showed that the presence of wolves in a chase area during wild boar (*Sus scrofa*) hunting sessions was significantly higher in the nights just after a chase ($P<0.01$, χ^2 test; $P<0.07$, exact Fisher test). The data obtained from genetics and camera trapping were consistent with one other, and in part complementary. The total cost of the study (c. 28,000 €) was moderate, suggesting that this integrated approach can be successfully used to monitor the structure and dynamics of local wolf packs.

Keywords Monitoring large carnivores · Canis lupus · Non-invasive genetics · Camera trapping · Wild boar hunting

Introduction

Wolf (*Canis lupus*) and other large carnivore populations, such as the brown bear (*Ursus arctos*) and the Eurasian lynx (*Lynx lynx*), have been threatened for centuries in Western Europe by persecution, deforestation and over-hunting of their natural prey (Breitenmoser 1998). Recent socio-ecological changes, legal protection and improved conservation programmes have reversed the declining population trends, at least for a few species in some regions (Boitani 2003). Wolves are now expanding and recolonizing parts of their historical range in Italy (Fabbri et al. 2007; Lucchini et al. 2002), mainly thanks to the expansion of

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forests in mountain areas and to the increase of wild ungulate populations (Randi 2005). However, the return of wolves in rural areas yields conflicts with farmers, who are no longer in the custom of defending their livestock from predators (Breitenmoser 1998). Uncontrolled poaching is thought to kill about 20% of the wolf population every year in Italy (Ciucci et al. 2007; Lovari et al. 2007). Thus, sound action plans should be implemented aimed at reconciling the coexistence of viable wolf populations with livestock farming.

Wolves are elusive and live at low density in widespread mountain areas in the Italian Apennine and Alps. Wolf packs, composed of the dominant breeding pair plus their offspring and non-reproducing, unrelated individuals, are territorial, but high mortality rates might deeply affect their composition and stability (Mech and Boitani 2003). Thus, even approximate information on pack number and location, home ranges, individual survival rates, and dispersal ranges is difficult and expensive to obtain. Field techniques traditionally used to assess species' presence and distribution (presence marks, snow-tracking, wolf-howling, radio-tracking) are labour-intensive, difficult to plan and can sometimes lead to evaluation errors (Genovesi 2002; Gese 2001). For instance, snow-tracking depends on the presence of snow, which is not always guaranteed in the Apennine. Free-ranging dogs and hybrids often occur within the wolf range (Randi and Lucchini 2002; Verardi et al. 2006), but field monitoring methods are seldom able to identify reliably the species, be they wolf, dog or hybrid. However, new methods such as non-invasive genetic sampling (NGS) and camera trapping have been recently applied to large predator monitoring programmes, and promise to solve these problems (Karanth and Nichols 1998; Kohn and Wayne 1997; Marucco et al. 2009; Sarmento et al. 2009; Taberlet et al. 1997; Waits and Paetkau 2005).

NGS is based on genetic identification of species, individual genotype and sex of unknown biological samples (mainly scats and hairs), which are collected without any interaction with the animals, and has been successfully applied in monitoring programmes of elusive species like the wolf (Fabbri et al. 2007; Lucchini et al. 2002; Marucco et al. 2009), brown bear (Gervasi et al. 2008; Taberlet et al. 1997), lynx (Mills et al. 2000a; Palomares et al. 2002) and wolverine (Brøseth et al. 2010). The target DNA is often scarce, fragmented or contaminated, and might generate genotyping errors, due to the amplification of false alleles (FA) or to allelic drop-out (ADO) (Pereira et al. 2009). These errors can be minimised by a multiple-tube approach (Taberlet et al. 1997) and statistical validation of the results of PCR replicates (Miller et al. 2002; Valière 2002). In this way, non-invasive genetics can provide data that could not be obtained by any other monitoring method (Waits and Paetkau 2005).

Camera trapping has been recently used to assess presence–absence in a number of carnivores (Cutler and Swann 1999; Gompper et al. 2006; Karanth and Nichols 1998; Moruzzi et al. 2002; Rosellini et al. 2008), as well as population dynamics (Karanth et al. 2006), but, surprisingly, it has rarely been applied to wolves (Berzi and Groff 2002; Palumbo et al. 2010; Spalton 2002). Camera trapping is less invasive (Van Schaik and Griffiths 1996) and less expensive (Seydack 1984) than other field methods, and can be used to document behavioural patterns that are difficult to observe directly (Kucera and Barrett 1993). Trapping success can be maximised by locating cameras along known wolf trails and in the most frequently marked crossroads (Barja et al. 2004; Vilà et al. 1994), rather than in randomly predefined cells. An individual's physiological (i.e., pregnancy, lactation) or pathological states (i.e., the presence of ectoparasites as *Sarcoptes* mites; Kreeger 2003) can be assessed using high-definition camera equipment. Camera trapping is limited by low species-specificity, and the identification of individuals is often difficult or impossible (Oliveira-Santos et al. 2009), especially in wolves (Genovesi 2002).

Wolf distributions in the northern Italian Apennines and western Alps are monitored through long-term programmes, mainly by the extensive use of NGS (Fabbri et al. 2007; Lucchini et al. 2002; Marucco et al. 2009). This study was planned to compare and cross-evaluate the performances of NGS and camera trapping in a small study area: the Corno alle Scale Regional Park (Fig. 1). The park hosts a variety of ungulates (wild boar *Sus scrofa*, roe deer *Capreolus capreolus*, red deer *Cervus elaphus*, mouflon *Ovis aries*) and other potential wolf prey species, and has been an important area during the early stages of wolf expansion along the northern Apennine ridge (Fabbri et al. 2007). The stable presence of wolves has been documented since the late 1990s by wolf-howling sessions, with evidence of reproduction events from 1999 to 2005 (Caniglia et al. 2010a). The NGS monitoring carried out previously using the same markers used in the present study (Caniglia et al. 2010b) led to the identification of three different wolf genotypes that were occasionally sampled within the park in 2002–2006, and four different genotypes that were found around the park. Based on these preliminary data and on the estimated size of wolf packs in Italy, varying from 2 to 10 individuals (Apollonio et al. 2004; Caniglia et al. 2010a), the whole study area was thought to host from one to three different packs. In this study, we aimed to (1) compare the results obtained by NGS and camera trapping methods, specifically by (a) counting the minimum number of wolves and packs in the Corno alle Scale Regional Park area; (b) reconstructing pack locations, composition and genealogies; (c) documenting the reproductive events; (d) identifying the possible presence of dogs

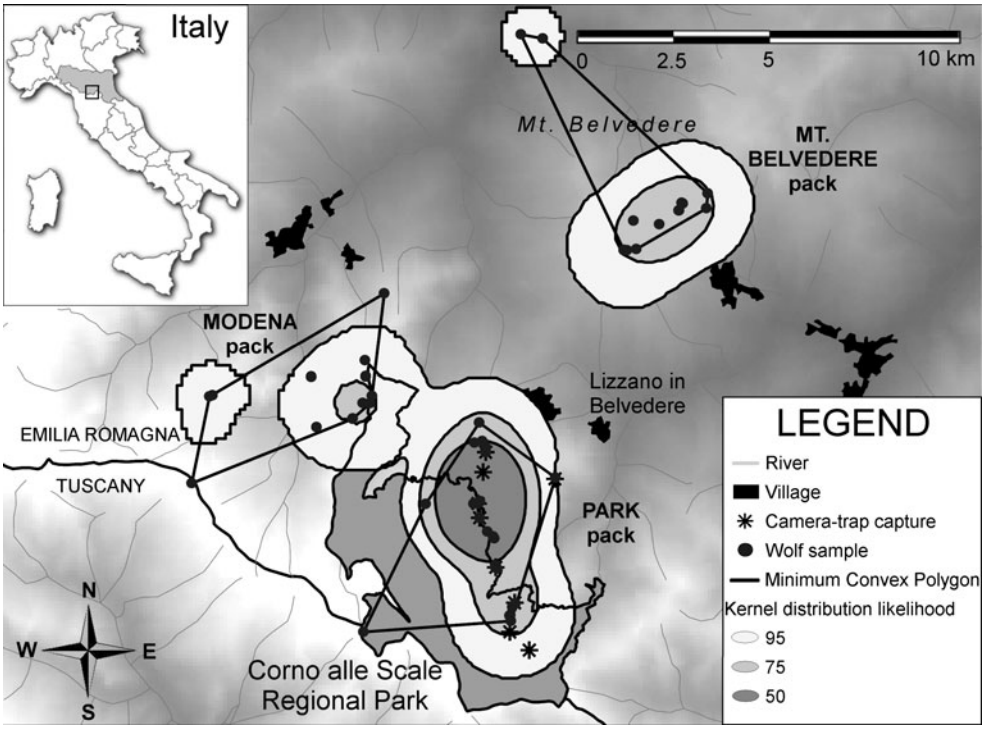

Fig. 1 Study area, location of collected samples and camera traps. *Areas where genotypes overlap within, but not across one another*, are surrounded by minimum convex polygons and are purported to host three different packs ('Park', 'Mt. Belvedere' and 'Modena' packs). This hypothesis was confirmed by mapping the contours of a kernel likelihood distribution (95%, 75%, 50%) of wolf samples

or wolf vs dog hybrids; and (e) obtaining information on wolves' health conditions. (2) Hunting is allowed in some areas around the park. Thus, we dedicated some specific camera trapping sessions to investigate the interactions among party chases of wild boars and wolf presence. Assuming that wolves are disturbed by hunting activities (Anwar et al. 2009; Nilsson 2003), we expected lower contact frequency during hunting compared to non-hunting periods. (3) Finally, we accurately evaluated and compared the costs required for both NGS and camera trapping methods.

Study area

The Corno alle Scale Regional Park (CSRP) covers a surface of 49.74 km^2 (including 21.17 km^2 of buffer area) in the northern Apennine, ranging from 500 to 2,000 m a.s.l. (Fig. 1). Oak and beech forests cover most of the territory, and typical alpine habitats are present in the upper ridges. The protected area includes roads and five villages, with an average human density of 28 people/km^2. Husbandry was relevant in the past, but nowadays, the number of livestock is strongly reduced. Hunting is allowed in some buffer areas. Viable populations of large ungulates (wild boar, roe deer, red deer, mouflon, fallow deer *Dama dama*) are present, with a higher density of wild boar and roe deer. Wandering wolf individuals were present from the 1980s, but evidence of a reproducing pack was obtained only in the late 1990s (Caniglia et al. 2010a).

Methods

Non-invasive genetic sampling

Biological samples for genetic analyses were collected from within the park, in the buffer areas, and in a northern surrounding area of c. 35 km^2 (Mt. Belvedere; Fig. 1). We defined eight fixed trails, for a total length of 25.7 km, roughly covering the entire range of wolf presence and selected to maximise the probability of finding fresh faecal samples. We performed 3–5-day sessions, once a month, for three winter months (December, January and February), during two winter seasons (2006–2007 and 2007–2008), for a total of 154.2 km (Table 1). Each trail was surveyed once per session to ensure a constant sampling effort. Sampling was focused in winter and during short periods, in order to maximise the genotyping success rates due to better DNA preservation (Lucchini et al. 2002; Santini et al. 2007). We collected only faecal samples that were judged fresher than 2 weeks, based on external appearance, exposure of deposition site and local weather conditions (Santini et al. 2007). Urine, hair and blood traces were also collected. Wolf snow tracks were followed until the first collection of any biological sample. The coordinates of every sample were recorded on a 1:25,000 map, and then digitalised on a GIS (ARCVIEW v.3.1, ESRI Inc, Redlands, California, USA). We froze the samples for 10 days at −80°C to kill parasites and *Echinococcus* eggs, and then stored them at −20°C in 95% ethanol until DNA extraction. All the collected samples were used as wolf locations to calculate

Table 1 Features of the selected sampling trails and number of samples collected per kilometre, with a constant sampling effort, in six sessions (December, January and February 2006–2007 and 2007–2008)

Trail	Length (km)	Altitude a.s.l. (min–max)	Collected samples	Samples/km
1	5.8	933–1,358 m	19	0.55
2	1.8	1,160–1,220 m	3	0.28
3	2.4	1,016–1,274 m	4	0.28
4	3.5	1,180–1,329 m	2	0.1
5	3	970–1,274 m	19	1.06
6	4.2	933–1,282 m	12	0.48
7	2.5	1,190–1,355 m	3	0.2
8	2.5	980–1,030 m	16	1.07
Total	25.7	933–1,358 m	78	
Average	3.2±0.47	1,145 m	9.75	0.51±0.04
Snow-tracks	2.6	980–1,358 m	25	9.61

Samples collected in occasional snow-tracking sessions have also been included

95%, 75% and 50% kernel spatial distribution likelihood (Worton 1989), determined by a fixed kernel method with smoothing factor (bandwidth) calculated by least-squares cross-validation (Kernohan et al. 2001; Seaman et al. 1999) using the Home Range extension for ARCVIEW v.3.x (Carr and Rodgers 1998).

DNA analyses

We extracted DNA using the Qiagen DNeasy Blood and Tissue Kits (QIAGEN) with a robotic liquid handling system MultiPROBE II^{EX} (PerkinElmer) in a room dedicated to non-invasive genetics, always adding blank controls (no DNA in PCR) to check for possible contamination. Wolf DNA was amplified using a multiple-tube approach (Taberlet et al. 1997) and a panel of 12 canine microsatellite PCR primers (FH2004, FH2079, FH2088, FH2096 and FH2137, Francisco et al. 1996; CPH2, CPH4, CPH5, CPH8 and CPH12, Fredholm and Winterø 1995; C09.250 and C09.253, Ostrander et al. 1993) as in Randi and Lucchini (2002), at the optimal PCR conditions for each primer (details available upon request). A quality screening was performed using three loci (FH2096, FH2137 and CPH8). Each sample was independently amplified four times per locus. Only those samples with a successful PCR rate >50% were further amplified at the other nine loci. The probability-of-identity, computed with these 12 loci on a reference Italian wolf population (Lucchini et al. 2002) and assuming that the samples originated from sibling individuals (Waits et al. 2001), was $PID_{sibs} \leq 0.01$, indicating the power to distinguish individuals, though strictly related, without ambiguity. The DNA products were analysed in an automatic sequencer ABI 3130xl (Foster City, California) using the ABI software GENEMAPPER v.4.0.

We performed a reliability analysis using RELIOTYPE (Miller et al. 2002). Unreliable loci (at threshold $R=0.95$) were additionally replicated another four times. All samples that were not reliably typed at all loci after eight PCR replicates were finally discarded. Consensus genotypes were reconstructed using the software GIMLET v.1.3.3 (Valière 2002; http://pbil.univ-lyon1.fr/software/Gimlet/gimlet.htm). GIMLET was used to match all the genotypes with a large Italian wolf dataset, obtained using the same methods and panel of loci. The Italian wolf database consists of multilocus genotypes obtained from DNA extracted from wolf carcasses collected throughout the entire Italian wolf range distribution in the last 15 years ($n=417$), from non-invasive samples collected during a monitoring project in the Apennine from 2000 to 2009 ($n=341$; Caniglia et al. 2010b, c) and in the western Alps from 1999 to 2004 ($n=130$; Fabbri et al. 2007).

We then used STRUCTURE v.2.2 (Falush et al. 2003) to identify individuals of hybrid origin (Randi and Lucchini 2002). As a reference, we included the genotypes of a baseline wolf and a baseline dog population, respectively composed of a random subset of 176 tissue samples from the previously cited database, and 118 blood samples collected from dogs living in Italian rural areas. STRUCTURE was run for 130,000 iterations (including 30,000 burn-in), with the 'admixture' model and assuming independent allele frequencies.

We analysed the number and distribution of genotype mismatches by MM-DIST (Kalinowski et al. 2006). The sex of every sample was identified by a Restriction Fragment Length Polymorphism analysis of the X-linked ZFX gene (Lucchini et al. 2002). Finally, we reconstructed the genealogy of the individuals through a maximum-likelihood approach implemented in COLONY v. 2.0 (Wang and Santure 2009), considering all the individuals as candidate parents. COLONY was run with allele frequencies and PCR error rates as estimated from the whole reference population, considering a probability of including fathers and mothers in the candidate parental pair of 0.5. The best genealogies reconstructed by COLONY were then verified in PARENTE v. 1.2 (Cercueil et al. 2002), and only highly

matching parent–offspring combinations were retained (up to 1/24 allele disparity, corresponding to a match >95%).

Camera trapping

Within the park, we used the same fixed sampling trails to place up to five cameras that were simultaneously active in the field. We placed the camera traps at crossroads or close to recent wolf faecal marks, avoiding the use of chemical or biological baits. Each camera-trapping session lasted from three to 30 consecutive days, for a total of 1,250 trapping-nights. We often relocated the cameras to maximise the detection rate and strategically placed them: (i) along trails used by wolves, (ii) at frequently marked crossroads, (iii) along obligatory passages between different areas in the predicted home range, or (iv) close to recent faecal or urine marks along the sampling trails that other wolves would probably check. We used both analogue (Fototrap Natura Service, http://www.fototrap.com, Forlì, Italy, with 400 ASA colour films) and digital devices (Fototrap Natura Service, with photo camera Sony P43, P600 or W35), activated by passive movement detectors (PIR) with a reaction time shorter than 1 s and assisted by visible flash lamps. Infrared LEDs were tested only in a preliminary session, then abandoned because of the low image quality. Camera traps were placed between 30 and 100 cm above ground level, with a default focus distance setting of 3 or 5 m. They were activated only at night-time, to save batteries and to maximise capture probabilities, since wolf activity in Italy is mainly nocturnal (Ciucci et al. 1997). We estimated nocturnal photo-captures peaks (Van Schaik and Griffiths 1996) from the detection times recorded in digital photos, always referring to the solar time. The consequences on wolf presence close to hunting sessions of wild boar, which occurred during winter periods, were assessed by placing from one to three camera traps close to the ending point of the chases, then contrasting the number of photo-captures in hunting vs non-hunting occasions.

Cost estimate

We analysed the costs of the two methods—camera trapping and non-invasive genetics—used in the monitoring study. For each method, we computed the costs of the equipment and the number of months needed in order to complete the work, multiplied for the average monthly (gross) salary for trained personnel. Camera trapping required photo trap devices—including batteries and movement detectors and film printing. The genetic costs were computed considering a unitary genotyping cost per PCR replicate (inclusive of all the reagents and machine usage), multiplied for all the replicates required by the high-quality multiple-tube protocol.

Results

Genetic identifications

We collected 103 non-invasive biological samples (40 in the first and 63 in the second winter season): 83 scats, 16 hairs, 3 urine traces on snow and 1 blood trace on snow, with an average of 0.51±0.04 SE samples/km along the transects (Table 1). We successfully genotyped 50 samples (48.54%), which obtained reliability scores $R \geq 95\%$: 35 from scat, 13 from hair, 1 from urine and 1 from blood. In these samples, the average number of PCR amplifications per locus was 4.5; the error rates were ADO=20% and FA=1%, on average. The consensus genotypes were grouped and assigned to 17 distinct individuals. After STRUCTURE analyses, two of these genotypes (4%) were assigned to the baseline dogs, and no individual was identified as a wolf vs dog hybrid. The other genotypes were identified as 15 distinct wolves: eight were sampled in the first season (labelled as F1, M1, M2, F2, F3, M3, M4, F4, where 'F' indicates female and 'M' male individuals), and 10 were sampled in the second season (F3, M3, F4, M5, M6, M7, M8, M9, M10, M11). Only wolves F3, M3 and F4 were present in both seasons (Fig. 2). The sex ratio was 2.75 M:1.00 F. The cumulative probability-of-identity observed with 12 loci was PID=0.0000003, or PID_{sibs}=0.001. The match probability (probability of one genotype to be generated a second time by chance = PID × population size) was P_M=0.000004, lower than P_{Msibs}=0.015 computed considering all the individuals to be related. A mismatch analysis by MM-DIST (Kalinowski et al. 2006) showed that these wolf genotypes differed from each other by at least three alleles. Thus, the probability of a 'shadow effect' (Mills et al. 2000b) was very low.

These genotypes were matched to those identified using the same genotyping protocol and the same panel of

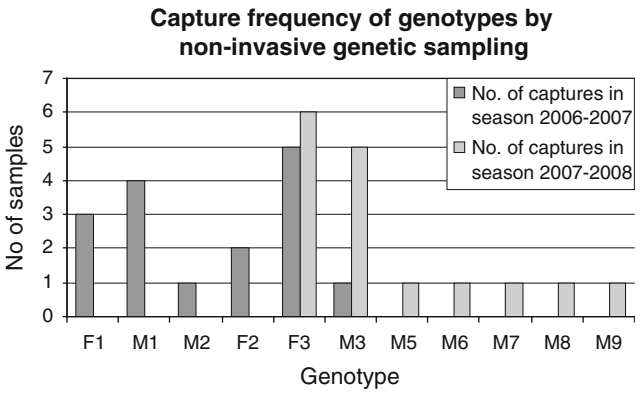

Fig. 2 Number of samples per wolf genotype within the park area in 2006–2007 (*black bars*) and 2007–2008 (*grey bars*). The most frequently sampled ($n \geq 3$) genotypes turned out to be the members of the breeding pairs (Fig. 3)

microsatellites during the NGS monitoring project conducted in the whole northern Apennine (c. 10,000 km^2; Caniglia et al. 2010b). Seven of these were already present in the database: two of them (F1, M1) were previously collected within the park boundaries (the third genotype previously detected was not found any longer), and four others (M4, F4, M10, M11) had been collected around the park and one (M3) more than 50 km east from the park. Mapping the spatial distributions of all the 15 wolf genotypes, we identified three areas where the genotypes overlap within, but not across one another (Fig. 1) as represented by minimum convex polygons (MCP), suggesting that we have sampled three different wolf groups respectively in (1) the eastern 'Modena' area, where genotype M4 has been sampled in the same location of genotype F24, which was identified during the NGS project as the breeding female of a stable pack since 2003 (MCP area=11.4 km^2); (2) the northern 'Mt. Belvedere' area (with genotypes F4, M10 and M11) (MCP area=9.7 km^2); and (3) the 'Park' area (MCP area=15.3 km^2), where the spatial distributions of the other genotypes closely overlap. This hypothesis was confirmed by mapping the contour areas of 95%, 75% and 50% kernel likelihood of NGS wolf locations. These results underline the presence of a core region (kernel 50%, 5.7 km^2) within the protected area, where the majority of samples has been found. In the 'Park' pack, we successfully identified from one to 11 samples for each genotype (Fig. 2), with an average of 2.9 samples per genotype.

Based on the allelic compatibility at the 12 microsatellite loci and using the software COLONY, then confirmed by PARENTE, we reconstructed only one putative 'Park' pack pedigree for each study season (Fig. 3). In 2006–2007, genotypes M1 and F1 were identified as the only possible parents (the breeding pair) of all the other genotypes (M2, F2 and F3), with no incompatibilities between them, except M3. This genotype, indeed, was previously sampled (Caniglia et al. 2010b) in 2005 in the Foreste Casentinesi, Mt. Falterona e Campigna National Park, a large protected area located more than 70 km from CSRP. Thus, wolf M3 could be an individual in dispersion. In 2007–2008, the breeding pair apparently changed, because wolves M1 and F1 were not sampled any longer. Additionally, four of the five new genotypes identified in 2007–2008 could have been generated (12/12 loci matching) by a new breeding 'Park' pack pair, namely composed of the wolves F3 and M3 (Fig. 3). The other genotype (M7) was not compatible with this breeding pair, but could be an additional offspring of the M1–F1 pair (with a single allele discrepancy), and thus was not sampled in the previous year. The average Queller and Goodnight (1989) relatedness between individuals in the 'Park' pack is r=0.58, hence coherent with the value expected for a familial group (Lucchini et al. 2002).

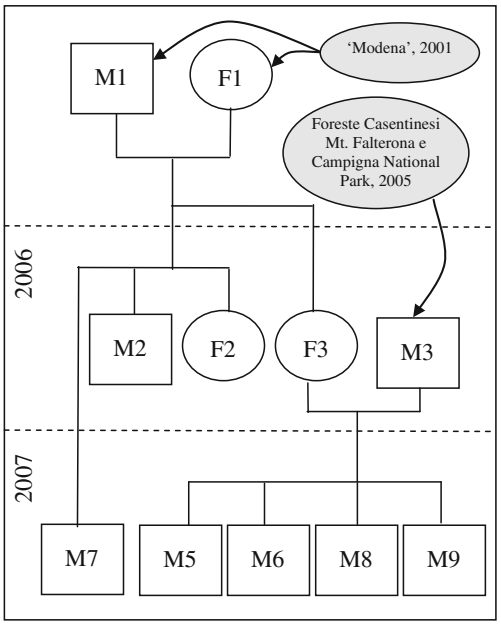

Fig. 3 The 'Park' pack pedigree in 2006–2007 and 2007–2008, as reconstructed by COLONY and confirmed by PARENTE on the basis of allelic compatibility among genotypes. In 2006, wolves M1–F1, which were already present in the park territory, are the only possible breeding pair, fully compatible with three putative offspring (genotypes: M2, F2 and F3). Female F3, however, should have been born before 2006, as she apparently gained the rank of breeding female in 2007. M3 is the only non-compatible genotype and, according to a long-distance (>70 km) dispersal event, originated outside the park. In 2007, wolves M3 and F3 are the only possible breeding pair, fully compatible with putative offspring M5, M6, M8 and M9, whereas M7 could be an additional offspring of the previous year. This is consistent with the camera trapping results that show at least three newborn pups in 2007

Camera trapping

Camera trapping provided 103 wolf pictures that were obtained during every season of the year, with 8% frequency of capture (total number of photos divided by trapping-nights). The peak in the number of captures was between 21.00 and 01.00, when 71% of total detected wolves were photographed (Fig. 4). The average number of wolves in a single photo was 1.26, with 14 images portraying 2 wolves, 2 images 3 wolves, and 3 images 4 wolves. In more than 56% of the images, it was possible to identify the wolf's sex, and whether the individual was a juvenile. The sex ratio deduced from the photos (number of independently detected individuals with recognisable sex) was 1.3 M:1 F, different from the sex ratio obtained by genetic data (P=0.01, χ^2 test). Many other mammalian species were photographed (from the most to the least common: wild boar, red fox *Vulpes vulpes*, hare *Lepus europaeus*, roe deer, stone marten *Martes foina*, badger *Meles meles*, fallow deer, red deer, mouflon, red squirrel *Sciurus vulgaris*, crested porcupine *Hystrix cristata*),

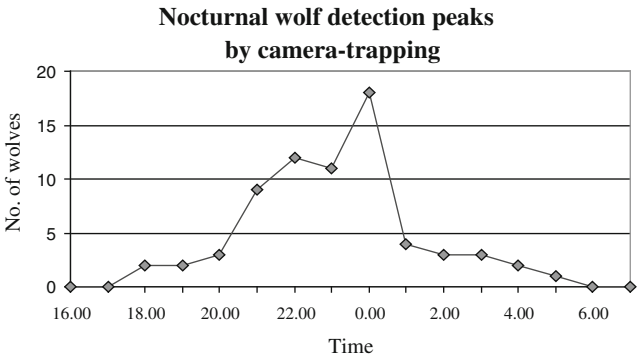

Fig. 4 Camera capture peaks of wolves during the night, expressed as number of wolves photographed per hour (consecutively portrayed individuals have been considered as a single capture)

including stray or wandering dogs, which were never pictured in association with wolves in mixed groups. Concordant with the genetic data, there were no individuals showing hybrid phenotypic features. The identification of individual wolves was rarely possible because of the absence of reliable distinctive markers. However, digital cameras produced a higher quality of photos than analogue cameras, permitting the detection of important details about sex and physiological states. Careful examination of the images allowed us to identify (a) a minimum number of four wolves that were present in the area at the beginning of the study (autumn 2006); (b) a reproduction event in 2006 (two juveniles were photographed in October), in 2007 (lactating female captured in May, Fig. 5a, and three juveniles in August, Fig. 5b) and in 2008 (post-lactating female photographed in July and two juveniles in August); and (c) a minimum number of five adult and two juvenile wolves at the end of the study (summer 2008). All the wolves showed the typical coat colour pattern of the Italian population, which is grey with black tips on the tail and ears, and a black strip on the fore paws (Altobello 1921). The apparent health conditions, as described by photos, were good, with the exception of a wounded animal and three wolves (including a newborn pup in 2008) with evident skin disease, which appeared infested with mange ectoparasites (*Sarcoptes* sp.). The minimum number of individuals identified from NGS and camera trapping was concordant, although not coincident (Table 2). In every season, genetic approaches were able to detect a larger number of individuals, resulting in one (2006–2007) or two (2007–2008) more wolves than detected by the camera traps.

During the wild boar hunting season of 2006–2007, one to three camera traps were activated for a total of 29 nights close to a meeting point of collective chases of wild boar. We obtained independent photo captures of wolves in four different nights (a single wolf in three nights, three wolves in one night; the average capture rate was 0.14 per night),

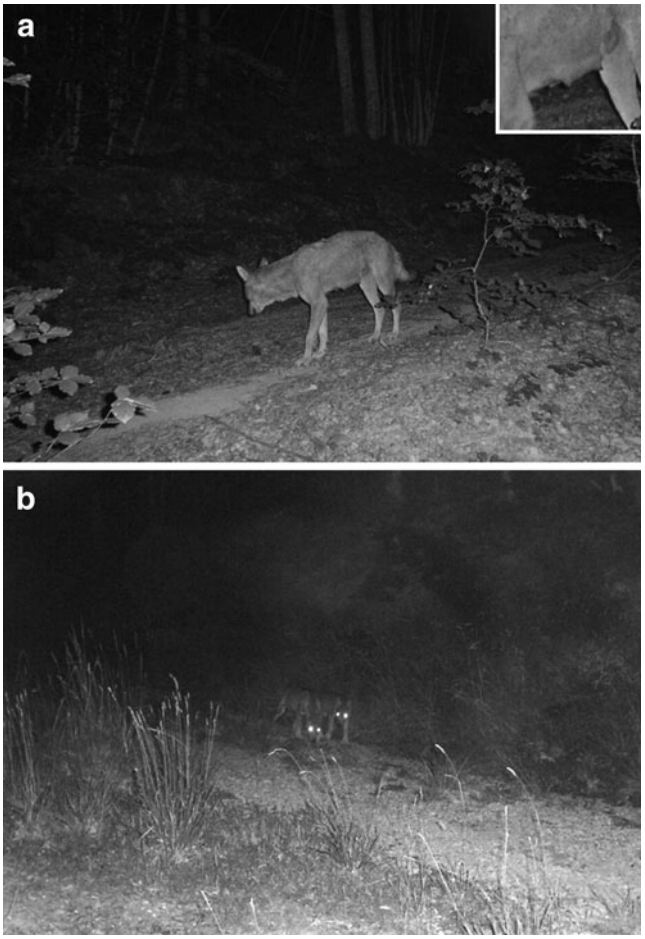

Fig. 5 **a** Photo capture of the breeding female wolf F3 on 28 May 2007, at the end of the reproductive period, apparent from the highly developed mammary glands (see detail). This reproduction event was confirmed by the photo capture of the offspring some weeks later. **b** Photo capture of two young wolves on 15 August 2007 (a third one was photographed a few seconds before). These sightings may indicate a home site, corresponding to that of the breeding female F3, in close proximity

but only after a chase. Wolves were never photographed during non-hunting nights. We tested a null hypothesis of constant capture probability in the 'hunting nights' and 'non-hunting nights', using a χ^2 test, which was discarded

Table 2 Minimum number of wolves, including yearlings, detected by non-invasive genetic sampling and camera trapping during three study seasons

Season	NGS	Camera trapping	Difference
2006–2007	6 (3 Y)	4 (2 Y)	2
2007–2008	7 (4 Y)	6 (3 Y)	1
2008–2009	–[a]	7 (2 Y)	–

Y yearlings

Each season starts according to wolf reproductive period, assumed to be every year at the beginning of May

[a] Biological samples collected in 2008–2009 were not yet analysed

($P<0.01$). The probability value computed using an exact Fisher test was $P=0.07$. In the following hunting season, however, adverse weather conditions did not allow us to collect additional significant data, with a single wolf photo capture in a non-hunting night.

Cost estimate

The total costs of the study are presented in Table 3. Camera trapping sessions required five camera traps, about 3 months of one-person work on the field; the NGS sessions needed 1 month of one-person work, which was totally overlapping with the camera trapping sessions, and thus performed by the same operator. The genetic analysis involves the costs for completing the 3,108 PCR amplifications (from the analysis of 103 samples×13 loci×n replicates per sample), plus 2 months of one-person work. The total study costs therefore sum up to c. 28,000 €.

Discussion

Formal comparisons of camera trapping and non-invasive DNA sampling have been performed only in a few recent studies (Rosellini et al. 2008; Sarmento et al. 2009), which were not aimed at identifying single individuals. Harrison et al. (2002) applied these methods to study a swift fox (*Vulpes velox*) population, but they used artificial tags to mark the individuals and did not apply robust multiple-tube protocols to avoid genotyping errors. Our study shows that non-invasive genetic sampling and camera trapping produced coherent results, allowing our key questions to be answered with certainty. We identified the number of wolf packs in the area, mapped their locations, and described their composition and genealogy.

The study area we investigated covered 85 km^2. The wolf territory size in Italy is widely variable from 80 to 300 km^2 (Apollonio et al. 2004; Ciucci et al. 1997), with an average of c. 100 km^2 (Corsi et al. 1999). Accordingly, given the strongly fragmented topography, the study area could match with the territory of one pack. In fact, we identified only one wolf pack within the park and two distinct packs around it, as indicated by the NGS data and confirmed by the kernel distribution analysis. We obtained estimates of the minimum number of wolves that were present in the park area during the two winter seasons 2006–2007 and 2007–2008. According to both methods, a minimum of six or seven wolves were permanently present, including two to four yearlings. These results were confirmed by additional camera trapping carried out in 2008–2009, when NGS was not performed, which documented the presence of seven wolves, including two yearlings. Additional genotypes were sampled in the 'Modena' and 'Mt. Belvedere' areas, outside the CSRP boundaries, where only the genetic survey was performed. The minimum number of wolves identified from NGS and camera trapping in the 'Park' pack was concordant, although not identical. In each season, genetic analysis was able to detect one (2006–2007) or two (2007–2008) more wolves than the number detected by cameras. This discrepancy could be due to the problematic identification of individuals by camera trapping. Wolves usually do not show conspicuous variable facial markers (Mech 1970), and we did not observe variation in coat colour patterns in the studied population. Hence, without the use of artificial tags, individuals cannot be reliably identified (Oliveira-Santos et al. 2009). Moreover, even though the members of a pack tend to move together (Mech and Boitani 2003), the low number of wolves portrayed per photo (average=1.26) strongly reduces the possibility of counting the minimum number of individuals occupying the area with a single or with consecutive shots. Consequently, although the total number of photos in respect to the supposed number of individuals is high (c. 9:1), a lower detectability of high-ranked individuals due to their higher awareness of the cameras (Larrucea et al. 2007) cannot be excluded, thus leading to a biased estimate. In the absence of reliable individual identification, Carbone et al. (2001) correlated the photographic rates to the species' density, but this estimate does not take into account all the factors influencing the capture rates, such as the probability of detection and the differences in movement rates, or spatial and temporal variables (Oliveira-Santos et al. 2009). In contrast, one of the main contributions of genetic tagging is to obtain reliable individual identifications (Lucchini et al. 2002; Waits and Paetkau 2005), which can be applied to large study areas for population size estimates (Cubaynes et al. 2010; Marucco et al. 2009). The multiple-tube protocol we used to genotype non-invasive wolf samples should reduce the risks of introducing false genotypes, and the PID

Table 3 Whole 2-year study costs for each monitoring technique. The total sum is inclusive of both the equipment and the months of work needed to complete the study

Technique	Field/lab work cost		Salary	Total
Camera trapping	Photo cameras	500 € × 5 = 2,500 €	2,000 € × 3 = 6,000 €	8,950 €
	Film printing	450 €		
NGS	PCR reagents and tools	4.8 € × 3,108 = 14,900 €	2,000 € × 2 = 4,000 €	18,900 €
Total cost		17,850 €	10,000 €	27,850 €

values were low enough to exclude the possibility that different individuals shared identical genotypes by chance ('shadow effect'; Mills et al. 2000b).

Camera trapping allowed the sex identification of some photographed individuals (in particular, lactating females were easily recognised by the size of their mammary glands), whereas molecular sexing allowed reliable sex identification of all genotypes. Yet, in our case study, the sex ratio obtained from camera traps was closer to 1 M:1 F than the sex ratio obtained from genetic identification (2.7 M:1 F). The sex ratio is alleged to be close to one (Kreeger 2003), even though there is some evidence of a possible bias towards males in adult wolves (Mech 1970) or towards female pups in low-density populations (Sidorovich et al. 2007). A slight prevalence of male genotypes has been detected in other NGS studies in Italy (Fabbri et al. 2007; Marucco et al. 2009). The skew in genetic data could result from male-biased marking behaviour, because scats are mainly used by males to mark the pack home ranges, and thus, they are more easily and frequently collected during NGS activities (Peterson et al. 2002). In this case, NGS and camera trapping can produce complementary results. In conclusion, it is likely that a minimum number of six–seven wolves has been permanently present in the park area during the last three winters, during which periods each dominant breeding pair would have started to delimit its home range prior to reproduction, and when each pack still includes some of the yearlings plus possible older offspring that did not leave the natal area (Mech and Boitani 2003). If we assume that sex ratio is close to one, as documented by camera trapping, we should also conclude that a few female genotypes (one or two) have been missed in the non-invasive genetic sampling.

Only four genotypes were re-sampled more than three times during this study: M1, F1, M3 and F3. Interestingly, the inferred genealogies indicate that these are the only genotypes that could have reproduced each season in the 'Park' pack, being the only parents fully compatible with the inferred offspring. These findings indicate that the dominant, territorial members of the breeding pair probably mark their home ranges more actively than the subordinate members of the packs, or do so in more prominent sites to enhance their marks' detection (Barja et al. 2004; Peters and Mech 1975). Therefore, the efficient sampling of subordinate individuals could be problematic, thus requiring a sampling strategy that involves a deeper use of snow tracks or other methods in addition to fixed sampling trails.

The composition of the breeding pair completely changed during this study. The new breeding pair in 2007–2008 was composed of female F3, already present in the park in 2006–2007, and male M3, an immigrant from an area more than 70 km distant from the park. One of the main causes of this high turnover can be explained by the low annual survival rate of young wolves in Italy that varies from 0.24±0.06 (Marucco et al. 2009) to 0.66 (95% CI: 0.54–0.77; Caniglia et al., unpublished data). This is probably due also to the ongoing human persecution in the area, as confirmed by a found dead wolf encountered next to the park at the end of 2009 that was probably killed with a hatchet (Italian Forestry Corp, official communication). These observations, although very limited in time (two wolf reproductive seasons) and in space (a small study area), support results from other studies in showing that wolf pack territories can be stable in time, but that pack compositions are unstable in Italy (Apollonio et al. 2004) and elsewhere in Europe (Brainerd et al. 2008; Jędrzejewski et al. 2004, 2005). Although individual wolf and pack identifications are almost impossible using only photographs, the comparison of genetic and camera trapping evidence allowed linking some genotypes to some photographed wolves, as for breeding female F3, identified both from genetics and camera trapping, or for newborn individuals.

Both genetics and camera trapping identified the presence of stray dogs wandering within the study area. We obtained neither genotypes nor pictures of presumed wolf vs dog hybrids. These observations are interesting, because the CSRP is located along a sector of the Apennine where the presence of hybrids has been repeatedly documented (Lucchini et al. 2002; Randi and Lucchini 2002; Verardi et al. 2006). Recent findings describe a close correlation between black or darker-than-usual wolf coat colour and recent hybridisation or past introgression with dogs bearing a functional melanistic mutation at the *β-Defensin* gene (Anderson et al. 2009). Although the origin and the dynamics of introgression of the *β-Defensin* mutation are still uncertain, pending further research, anomalous wolf coat colours can be easily documented by camera trapping, thus producing relevant information and early warnings for deeper monitoring of hybridisation and introgression, also through genetic identifications.

Camera trapping has supplied interesting additional information on wolf behaviour during this study. The results surprisingly showed a significant increase in the number of wolves roaming close to a meeting point of collective chases of wild boar. These data can be explained by a combination of different factors: (a) the unusual passage of humans and dogs might lead wolves to intensify survey and marking of their territories; (b) the presence of game blood traces might attract wolves; (c) the presence of wounded wild boars that hunters cannot capture can attract wolves. In this case, wild boar hunting would ensure a reward to the wolves' behaviour. However Francisci and Guberti (1993) documented that wolves are most frequently killed at the beginning of the hunting seasons, suggesting that interactions with humans can also be dangerous. These

findings shed light on unusual wolf behaviours, and additional camera trapping observations could contribute to a better understanding of wolf interaction with human activities. We anticipate that a deeper knowledge of wolf behaviour, during active or opportunistic predation, could have significant applications in practical methods to prevent or limit livestock depredations (Ciucci and Boitani 1998; Cozza et al. 1996; Gazzola et al. 2008).

This pilot study required limited human effort in terms of number of people ($n \leq 2$), which is considerably lower than for other tracking methods (Genovesi 2002; Gese 2001). The two short winter sampling sessions produced many good-quality samples and were widely overlapping with camera trapping sessions. The total cost of the project was limited, considering the fine-grained and highly detailed results (individual identifications and familial relationship) that were obtained without causing any disturbance to the animals. As expected, the costs were higher for NGS than for camera trapping, but cheaper than other traditional methods for carnivore surveys based on trapping and radiotelemetry (De Barba et al. 2010; Schwartz et al. 2007; Solberg et al. 2006), and are therefore feasible for many deep-monitoring projects.

Conclusions

Monitoring wolves is often difficult because of their elusiveness and low density in the wild, but is also essential for designing and applying sound conservation programmes, which should include procedures aimed at limiting conflicts with human activities, such as hunting and husbandry. However, the costs of long-term monitoring projects of widespread wolf populations are rarely affordable. Non-invasive genetic sampling can be used to fulfil these needs. In this study, we showed that the integration of NGS and camera trapping provided concordant results, and generated a detailed reconstruction of the structure, short-term dynamics and physical health of a local wolf population, as well as the exclusion of any morphological or genetic signal of hybridization. Thus, the low human and economic efforts required to carry out this pilot study make this integrated approach widely applicable, in different areas and habitats, without disturbing the animals. The two methods are complementary and reciprocally supported, yielding data that can be cost-effective detailed components in wider monitoring projects, in addition to or in place of other traditional field methods.

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