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'Video-scats': combining camera trapping and non-invasive genotyping to assess individual identity and hybrid status in gray wolf

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Motion-activated video cameras and non-invasive genetic sampling are tools commonly used to obtain relevant information on wild populations of rare or elusive carnivores while minimizing disturbance. The two approaches are usually implemented separately, but they are occasionally integrated at a population level, mostly in order to estimate population size. Here we show the advantages of combining camera trapping and non-invasive genotyping at an individual level, in a monitored Italian wolf population affected by introgression from domestic dogs. After 24 defecation events recorded by camera traps located at marking sites, samples ('video-scats') were collected in order to determine the individuals' identity based on the analysis of sex markers, 11 autosomal microsatellites, two Y-chromosome microsatellites and the control region of the mitochondrial DNA. Genetic data for 19 successfully genotyped scat samples were combined with morphological and behavioural traits observed in the videos and compared to data from ongoing genetic monitoring, all of which enabled us to determine sex, pack membership, breeding status, morphological traits (including those used to assess hybridization), sampling history, and introgression level of each individual. Finally we discuss the advantages and possible drawbacks of 'video-scats', supporting their use as an opportunistic source of valuable data.

Camera trapping (CT) and non-invasive genetic sampling (NGS) have become common tools in monitoring wild populations of elusive or rare species. CT has proved especially valuable in documenting the presence of cryptic species (Linkie et al. 2013), estimating population size or density (Karanth et al. 2006, Sollmann et al. 2013), revealing significant behavioural traits (Harmsen et al. 2010), and providing high-quality images of specific individuals in the population (Courtney et al. 2015). On the other hand, the genotyping of non-invasively collected samples provides information on the presence and spatio–temporal distribution of individuals, thus revealing key data on the population (population size, social structure, genetic diversity, dispersal patterns, occurrence of hybridization and diseases, diet composition, etc., reviewed by Waits and Paetkau 2005).

The two techniques are currently widely implemented to monitor carnivore populations, especially in elusive or threatened species. In the last decade, these methods have been adopted to assist in the management of the gray wolf *Canis lupus* in many regions (Stenglein et al. 2010, Bohling and Waits 2011, Randi 2011, Scandura et al. 2011, Caniglia et al. 2012, Cohen et al. 2013, Ražen et al. 2016, Subba et al. 2017). Despite their wide application, though, both methods have limitations. NGS is based on non-invasively collected biological material (mostly faeces) and leads to taxonomic and individual identification (the so called 'genetic fingerprinting'), but does not facilitate the association of any additional information that requires visual data (like age class, morphological traits and social status) to the sampled individuals (Waits and Paetkau 2005). On the other hand, CT, which is often employed to monitor wolf presence, abundance or movements, is rarely used for the detection of individual traits (Ilemin 2014), mainly because of the very limited morphological variation among wolves, which has hampered the implementation of mark–recapture methods based on individual recognition. However, morphological variation is enhanced by genetic variation and is usually higher in presence of genetic admixture (as in hybrid zones of North America, for instance; Benson et al. 2012). In Europe, hybridization with domestic dogs has been documented in many regions (e.g. Italy – Verardi et al. 2006; Spain – Godinho et al. 2011, 2015; Bulgaria – Moura et al.

2014; and the Baltic countries – Hindrikson et al. 2012), and it is possibly leading to an increase in inter-individual variation as a consequence of the introgression of domestic genes into the wild population. Under such circumstances, the morphological identification of individuals may become easier.

Nonetheless, the spread of domestic genes in wild wolf populations poses severe conservation concerns. Maladaptive gene variants, in fact, may be introduced into wild populations through backcrossing, causing fitness declines and outbreeding depression, occasionally even to the extent of driving local populations to extinction (Rhymer and Simberloff 1996, Galaverni et al. 2017).

While genetic tools proved useful to detect hybrids, their efficiency is hindered by the high dilution of domestic genes in backcrossed individuals (which represent the vast majority of hybrids), and by the uncertain assignment of the latter to parental populations (Lorenzini et al. 2014, Randi et al. 2014). In particular, the detection of hybridization through molecular markers such as microsatellites is reliable only up to 2–3 generations in the past (Randi 2008). Therefore, phenotypic characteristics are usually taken into account alongside molecular markers to assist with the hybridization assessment. However, while some specific features in wolves were shown to be the result of introgression from domestic dog (such as the black coat, white claws and spur on the hind legs, Galaverni et al. 2017), other traits (e.g. floppy ears, particular pelage patterns) are not currently considered as a striking evidence of such gene flow.

Furthermore, considering only morphological criteria for the detection of hybrids may be misleading because of the possible occurrence of 'asymptomatic' hybrids (i.e. showing a wild-type phenotype, Lorenzini et al. 2014) and because of the difficulty to distinguish between the effect of introgression and intra-specific phenotypic variation. As a consequence, integrating genetic and morphological data to assess hybridization at the individual level is strongly advised. Thus far, however, this has only been possible for captured or dead wolves.

In the present study, we show the potential benefits of combining the use of motion-activated video cameras and non-invasive genotyping to provide complementary information on individual wolves in an expanding wild population of central Italy. We sampled and genotyped wolves' scats left on the ground in front of an active camera (defined here as 'video-scats'), in order to determine sex, pack membership, breeding status, morphological traits and introgression level of each individual. Here we present the method, discussing its strengths, limits and applicability. We also suggest how to increase its efficiency and suggest possible applications to the conservation and management of wolf.

Material and methods

Study area

The study was conducted in a mountainous area located in Tuscany (Italy), including the massifs Alpe di Catenaia and Alpe di Poti (northwest to the city of Arezzo). Elevations range between 250 and 1414 m a.s.l. Land cover mainly comprises mixed deciduous hardwoods, dominated by oak *Quercus* spp., chestnut *Castanea sativa* and beech *Fagus sylvatica*. The wolf is the only large carnivore in the area, otherwise populated by wild ungulates such as the ubiquitous wild boar *Sus scrofa* and roe deer *Capreolus capreolus*, plus a limited number of red deer *Cervus elaphus*. Wolf presence in this area has been monitored since 1998, through summer sessions of wolf howling (Passilongo et al. 2010), NGS (Scandura et al. 2011) and, more recently, CT. In recent years, signatures of wolf–dog hybridization were detected in carcasses and non-invasively genotyped individuals (Iacolina et al. 2010, Bassi et al. 2017).

Remote camera trapping and video analysis

Camera trapping (CT) was conducted in the study area between March 2013 and October 2016, using a number of cameras ranging between 15 and 45, for a total of 10 183 trap days. Remote motion-activated cameras were placed at known scent marking sites, used by resident wolves and situated along dirt roads (mostly at crossing points). Each camera trap was active 24 h day^{-1}, 7 days $week^{-1}$, and was visited by observers at variable intervals (from 2 to 20 days) in order to change batteries and SD cards. No bait was used at trapping sites. Three models of built-in HD digital cameras were used: Buschnell trophy cam HD, UVision UV 562 and UV 572. All three cameras were provided with passive infrared sensor (PIR) and LED flash. Using these cameras, videos were recorded which lasted 60 s and were separated by 1-s intervals. After removing SD cards, videos showing wolves were labeled (combining consecutive videos, when wolves were filmed for more than 60 s) and carefully screened by one of the authors (L. Mattioli).

We considered a pack to be each social unit constituted at least by a territorial pair, regardless of its reproductive success. Alpha individuals in a pack were identified on the basis of their scent marking behavior (raised-leg urination by alpha male, flexed-leg urination by alpha female, and frequent ground scratching, Mech and Boitani 2003). For each individual, the following information was recorded whenever detectable: sex (by observation of the genital area, evidence of lactation/pregnancy, or posture during urination), age class (adult/pup), marking behavior, morphological anomalies possibly associated to dog introgression (e.g. coat color pattern or melanism, spur on hind legs, floppy ears, etc.) and other peculiar traits useful for individual recognition, such as tail shape and carriage. Once identified, alpha individuals became the focal animals of their pack, allowing for the distinction between neighboring packs as well as for the univocal assignment of most videos to a specific social unit. Finally, wolves that were video-recorded while defecating were identified and their pack membership was assessed along with their social rank (as either breeding adults or non-breeding pack members).

Genetic analysis

During each check of the cameras, the site was inspected and any scat collected. The collector wore sterile gloves and transferred a sample of a few centimeters from the scat into a 25-ml plastic tube, which was subsequently filled with 5–10

volumes of absolute ethanol and stored at room temperature. After careful checking of videos, when a defecation event was recorded, the age of the corresponding scat (i.e. the time elapsed between defecation and collection) was calculated, and the sample further processed only when its age was ≤ 12 days (because of the expected yield decrease, Santini et al. 2007). Genotyping was performed by NGB Genetics (Bologna, Italy), following the recommendations provided by Budowle et al. (2005) for animal DNA forensics.

DNA was isolated from scat samples using the Qiagen QIAamp DNA Stool Kit and following the manufacturer's instructions. Genotyping was based on the amplification, in three replicates, of one marker (Amelogenin gene) for gender determination and 11 unlinked autosomal microsatellites (dinucleotides: C09.250, CPH2, CPH4, CPH5, CPH8, CPH12; Ostrander et al. 1993, Fredholm and Wintero 1995; tetranucleotides: FH2004, FH2137, FH2088, FH2096, FH2079; Francisco et al. 1996).

Two multiplexed polymerase chain reactions were carried out. Multiplex 1 contained loci CPH4, CPH5, CPH12 and FH2079 and multiplex 2 contained the remaining loci. Samples were amplified in a 10-µl reaction mixture containing 0.2 µM of each primer, 4 µl of template DNA and 5 µl of Qiagen Multiplex PCR Kit. Amplification conditions consisted of an initial denaturation step for 15 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at either 56°C (multiplex 1) or 58°C (multiplex 2) for 60 s, and extension at 72°C for 60 s, and then a final extension at 60°C for 30 min. All the successfully amplified products were analyzed by capillary electrophoresis on an automated sequencer ABI PRISM 310 with an internal size standard (LIZ 500). Alleles were scored using GeneMapper 3.7. Samples successfully genotyped at autosomal microsatellites were sequenced once at 350 bp of the control region of the mitochondrial DNA (CR-mtDNA) following Vilà et al. (1999) and, when male, were also typed at two Y-chromosome microsatellites (MS34A and MS34B – Sundqvist et al. 2001) following Iacolina et al. (2010). Amplification (PCR) success was calculated as the percentage of successful single-locus PCRs.

For each scat sample analyzed, a consensus genotype was obtained by examining the three replicates, accepting as heterozygote any locus showing two different alleles in at least two independent repetitions, and as homozygote any locus showing one single allele in all three repetitions. Then, the resulting consensus genotypes were compared to those obtained, using the same methodology, during the previous genetic monitoring of the Arezzo population (i.e. 83 genotypes obtained by NGS or from carcasses recovered from 2005 to 2016). The occurrence of matches with genotypes already recorded in the area was tested using Gimlet ver 1.3.3 (Valière 2002). The probability of identity (i.e. the probability of occurrence of the same allele combination in a different individual in the population, expressed either as $p_{id[random]}$, i.e. calculated for random dyads in the population, or $p_{id[sibs]}$, i.e. calculated for full siblings; Waits et al. 2001) was calculated for each consensus multilocus genotype. If a match was found (supported by a $p_{id[sib]} < 0.01$), the 'video-scat' was regarded as a resampling of a known individual. Additional doubtful alleles obtained from the video-scat were confirmed only if occurring in the matching 'known' genotype, whereas, when they did not occur, they were excluded from calculations and regarded as missing data. Error rate for each sample was estimated as the number of incorrect single-locus genotypes divided by the total number of single-locus genotypes obtained. In doing so we considered as the 'correct' reference genotype either the matching genotype in the database, whenever possible, or otherwise the consensus genotype obtained.

CR-mtDNA sequences were compared with other published sequences using BLAST (<https://blast.ncbi.nlm.nih.gov>), in order to ascertain whether they matched with the diagnostic Italian wolf haplotype W14 (Genbank accession code AF115699; Randi et al. 2000), while Y-chromosome haplotypes were classified as arising either from the wolf or from the domestic dog population, according to Iacolina et al. (2010). This analysis was replicated to confirm the results, if either mtDNA or Y-chromosome haplotypes differed from the expected Italian wolf haplotypes.

Finally, a Bayesian analysis was performed in Structure ver. 2.3.4 (Pritchard et al. 2000) in order to assess the degree of introgression from domestic dog in the genotyped individuals and to associate it to the morphological traits detected. As reference 'wolves', we considered 30 genotypes from the same geographic area that had no sign of introgression at autosomal microsatellites, CR-mtDNA, Y-chromosome or morphology (obtained exclusively from carcasses). Similarly, 37 local domestic dogs from either private owners or kennels were included in the analysis as reference genotypes (popflag = 1). Genotypes obtained from scat samples were also included, with popflag = 0. Structure was run 10 times, with fixed K = 2 and 250 000 burn-in, followed by 250 000 iterations as data collection, admixture model, uncorrelated allele frequencies between populations and the option updating allele frequencies using only individuals with popflag = 1. The proportion of admixed ancestry of a given individual was inferred from its estimated membership to the wolf cluster (Q_w), calculated in the run with the highest posterior probability.

Results

Over a total of 10 183 trap days and 2172 videos of wolves recorded between 2 March 2013 and 5 October 2016, 65 defecation events were recorded with an average of one event every 156.6 trap days. Twenty-four scats were collected within of 12 days after defecation and sent to the laboratory for genetic analyses. Their age ranged between 16 h and 12 days. The remaining scats were not collected either because considered too old (> 12 days) or because the defecation act was not noticed during the preliminary on-site video checking.

A reliable multilocus genotype was obtained for 19 scats (79%) (Table 1). No case of pooled sample (possibly due to over marking) was observed. Amplification success ranged individually between 0% and 100% (on average, 76.9%) and was found to correlate to the sample age (Spearman's correlation coefficient, rho = –0.623, $p < 0.01$), while error rate ranged between 0% and 21.4% (on average 4.1% for successful samples). Almost all the errors detected (92%) were due to allelic dropout. The analysis in Gimlet revealed

Table 1. Results of multilocus genotyping of 24 'video-scats'. The following data are reported: sex (genetically-assessed by the Amelogenin gene), the consensus 11-microsatellite genotype (three repetitions), PCR success (%), error rate % (the number of incorrect single-locus genotypes divided by the total number of single-locus genotypes obtained)and the probability of identity associated to each multilocus genotype, calculated for random dyads ($p_{id[random]}$) and full sibling pairs ($p_{id[sibs]}$).

Sample	Genotype	Sex	CXX250	FH2004	FH2137	FH2088	FH2096	CPH02	CPH08	FH2079	CPH12	CPH04	CPH05	PCR success	Error rate	$p_{id[random]}$	$p_{id[sibs]}$
1	AP40	F	0/0	110/110	0/0	93/117	92/96	96/98	195/203	271/275	192/192	0/0	116/118	70%	2%	9.00×10^{-8}	2.64×10^{-3}
2	–	F	0/0	0/0	0/0	0/0	0/0	98/100	0/0	0/0	0/0	0/0	0/0	27%	–	–	–
3	–	–	0/0	0/0	0/0	0/0	0/0	92/92	0/0	0/0	0/0	0/0	0/0	21%	–	–	–
4	AP37	M	133/133	110/176	154/156	125/125	92/100	100/100	203/207	271/275	192/192	145/145	118/118	100%	3%	2.65×10^{-9}	5.70×10^{-4}
5	AP34	M	133/133	110/164	178/178	125/125	96/100	92/92	203/203	263/263	192/206	145/145	118/128	100%	3%	2.53×10^{-8}	8.28×10^{-4}
6	AP43	F	129/133	110/110	156/164	93/125	92/100	100/100	203/203	263/275	192/210	145/145	118/118	100%	0%	1.17×10^{-9}	3.57×10^{-4}
7	AP37	M	133/133	110/176	154/156	125/125	92/100	100/100	203/207	271/275	192/192	145/145	118/118	91%	2%	7.96×10^{-10}	3.41×10^{-4}
8	MF01	F	129/133	106/164	164/168	93/125	96/100	92/100	195/203	263/267	192/192	145/147	120/128	100%	0%	7.09×10^{-10}	1.78×10^{-4}
9	–	–	0/0	0/0	0/0	0/0	0/0	92/100	0/0	0/0	0/0	0/0	0/0	12%	–	–	–
10	MF03	M	133/133	106/176	168/178	93/125	96/100	92/100	203/203	263/263	192/192	145/147	128/128	88%	3%	1.23×10^{-9}	2.63×10^{-4}
11	MF04	M	133/133	106/176	168/178	117/125	96/100	92/98	195/203	263/263	192/192	147/147	118/120	97%	1%	1.02×10^{-10}	1.43×10^{-4}
12	AP11	F	129/133	110/164	164/168	93/125	96/100	92/100	203/209	263/267	192/210	145/147	116/118	85%	12%	2.30×10^{-7}	1.78×10^{-3}
13	AP37	M	133/133	110/176	154/156	125/125	92/100	100/100	203/207	271/275	192/192	145/145	118/118	100%	0%	7.96×10^{-10}	3.41×10^{-4}
14	MF03	M	133/133	106/176	168/178	93/125	96/100	92/100	203/203	263/263	192/192	145/147	128/128	100%	0%	1.23×10^{-9}	2.63×10^{-4}
15	MF05	F	133/137	110/110	160/178	93/125	92/96	92/96	203/203	263/271	192/206	145/145	116/118	100%	0%	4.57×10^{-10}	2.04×10^{-4}
16	AC55	M	133/133	110/110	160/178	117/117	92/100	92/98	195/207	263/267	192/206	145/147	118/118	97%	0%	2.02×10^{-9}	3.16×10^{-4}
17	AP51	M	133/133	110/164	154/164	125/125	92/96	92/100	207/209	267/271	192/210	145/145	116/118	94%	3%	5.79×10^{-9}	6.64×10^{-4}
18	AC60	F	137/137	164/164	160/178	117/121	96/100	100/100	201/203	263/271	192/206	145/153	118/128	91%	0%	4.69×10^{-11}	1.71×10^{-4}
19	AP11	F	0/0	110/164	164/168	93/125	96/100	92/100	203/209	263/267	192/210	145/147	116/118	88%	7%	1.71×10^{-8}	8.08×10^{-4}
20	–	–	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0%	–	–	–
21	–	–	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0%	–	–	–
22	AP57	M	133/133	164/176	154/168	93/117	92/96	100/100	203/207	263/267	192/192	141/141	128/128	97%	3%	1.46×10^{-13}	7.17×10^{-5}
23	MF04	M	133/133	106/176	168/178	117/125	96/100	92/98	195/203	263/263	0/0	145/147	118/120	97%	0%	1.72×10^{-10}	2.20×10^{-4}
24	MF03	M	133/133	106/176	168/178	93/125	96/100	92/100	203/203	263/263	0/0	145/147	128/128	91%	0%	3.32×10^{-9}	4.06×10^{-4}

that the 19 consensus genotypes corresponded to 13 individuals, 7 males and 6 females (Table 1), and 7 out of 13 were already 'known', i.e. they had been previously detected by the analysis of other non-invasive genetic samples in the area. $p_{id[random]}$ for them ranged between 1.46×10^{-13} and 2.30×10^{-7},whereas $p_{id[sib]}$ ranged between 7.17×10^{-5} and 2.64×10^{-3}. Therefore, each genotype had a negligible chance to be shared by other individuals in the population.

In 18 defecation events, the video enabled individual recognition and the scat was successfully analysed (95% of the 'video-scats', 0.8% of the total number of videos). Wolf identification by NGS matched that assessed by CT (Table 2): every time a given individual was visually recognized in more videos, the genetic analysis of the corresponding samples produced the same genotype. The wolves identified belonged to four packs and were filmed either alone or with one to three other individuals. Since trapping sites were located in correspondence of previously known marking points, an intensive marking behavior was shown by animals (Table 2). Most of the recorded wolves showed types of marking other than the fecal one: raised-leg urination and ground scratching being the most frequent ones. According to their dominance and marking behavior, most of the wolves (10 out of 13) were recognized as breeding adults (alpha). All of them except one (MF05) resulted from two or more fecal samples (AP37 and MF03 were represented three times in the 'video-scats') over a maximum of six consecutive years of genetic monitoring.

Most videos were nocturnal, thus making it more difficult to evaluate morphological anomalies that could be associated to hybridization with domestic dog. However, at least three individuals – genotypes AP11, AP51 and AC55 - clearly showed anomalous phenotypes (Table 2). Female AP11 (alias αF1-PS) and male AP51 (alias βM-PS) were members of the same pack and both had $Q_w < 0.90$, while male AC55 (identified as αM15-CN) showed $Q_w = 0.973$, but was carrying a canine Y haplotype (H03). Another individual (MF01, which was not identified by video analysis and not previously sampled by NGS) also showed a possible genetic signature of canine introgression ($Q_w = 0.872$), yet in absence of conspicuous anomalies in its morphology.

Discussion

The results of this study highlight the usefulness of the integration of simultaneous data from two so far disjoint sources of information for wolves (video recordings and genetic analyses of fecal samples). The combination of remote sensing and genetic analysis of non-invasive biological material is not new. In most cases, however, the two methods were used in the same area just to collect as much data as possible on local populations of elusive carnivores in a non-invasive manner (e.g. wildcat *Felis silvestris* in Italy; Anile et al. 2012, Velli et al. 2015). Galaverni and colleagues (2012) compared the information obtained by NGS and CT within the territory of a wolf pack in Italy. They outlined the complementary contribution of the two methods in providing information on pack composition and size as well as on morphology and genetic make-up. In those studies, however, the two methods merely were implemented in the same area, but were not actually integrated (i.e. they were used to obtain independent estimates of population density or pack size and not to collect complementary information on specific individuals).

Used separately, NGS and CT can actually provide information on local wolf populations. Their integration, however, ensured by the analysis of scats produced by visually identifiable individuals, has the potential to give full details on the structure of local packs and on the nature of breeding pairs (Fig. 2). In our study, in fact, the two approaches are shown to be mutually supportive for several purposes: 1) individual identification, which can be obtained by consistent observations of a peculiar individual phenotype (CT) and confirmed by non-invasive genotyping (NGS); 2) pack membership assessment, which is obtained by location, number of individuals observed, and focal animal characteristics (CT) and can be confirmed by kinship analyses (NGS); 3) identification of mates (alpha pair), recognized mostly from their behavior and occasionally through signs of female pregnancy/lactation (CT); it can be confirmed by the genotyping of the offspring and parentage analysis (NGS); 4) assessment of introgression of dog genes in individual genomes is made through genetic analyses (NGS), but can be confirmed by visual inspection of specific morphological anomalies, which can be indicative of a domestic ancestor (CT).

Moreover, all these data are obtained in a non-invasive fashion, without capturing nor even disturbing the animals. This can have strategic management implications: 1) a validated individual recognition allows for the application of visual capture–recapture density estimation, which in turn enables us to model the effects of temporal, environmental and individual covariates on density and on the way individuals use space; 2) pack structure and membership assessment provides information on pack stability over time; 3) pedigree analyses and information on packs size obtained by videos jointly allow for an assessment of reproductive success; and 4) genetic and morphological traits allow for the ascertainment of recent hybridization, giving the opportunity to identify target hybrid individuals that can be removed or sterilized.

Remarkably, our genetic analysis confirmed the identity of the individuals that were recorded more than once while defecating in front of a camera, and which had been initially identified by one of the authors (L. Mattioli) on the basis of morphological cues only. This is indeed promising, as it proves that intra-population phenotypic variation may allow for individual identification in wolves, which, in our study, was possibly facilitated by the introgression of canine genes at population level.

Identifying admixture patterns in such introgressed populations as the Italian wolf is far from trivial. Traditional non-invasive genetic surveys (based on a limited number of microsatellites, e.g. 12 or lower) cannot assess the introgression rate with confidence (Randi et al. 2014). Because of the dilution of the introgressed genes after a few generations of backcrossing with wolves (Randi et al. 2014), genetic tools cannot always be conclusive on the diagnosis of hybridization. The canine origin of some physical traits, on the other hand, is well documented (Galaverni et al. 2017) and morphology is therefore almost unanimously recognized as a fundamental tool for the detection of hybrids. For instance, the combined use of CT and NGS enabled us to identify two individuals (AP11 and AC55) – in distinct packs of our

Table 2. Information on the 'video-scats' analyzed and on the defecating individuals filmed by remote cameras. Wolf ID is the univocal code given to the individual recognized in the video (it incorporates information on social status – non-alpha individuals are conventionally indicated as β - sex and pack). Total nr. of individuals is the number of different wolves observed in the video. Marking behavior refers to known ways adopted by wolves to mark the territory (FM = fecal marking, RLU = raised-leg urination, STU = standing urination, FLU = flexed-leg urination, SQU = squatting urination, GSC = ground scratching). Qw is the probability to be assigned to the 'wolf' cluster, inferred by Structure software. The CR-mtDNA haplotype (W14 is the typical Italian wolf haplotype) and the two-microsatellite Y-chromosome haplotype (H01 and H02 are the most common in Italian wolves, while H03 in Italian domestic dogs; Iacolina et al. 2010) are also reported. Total samplings is the overall number of resamplings of the specific genotype over the whole genetic monitoring of the population. 'Sampling years' indicates the number of years, when such resampling occurred. nd = not determined.

Video analysis									Genetic analysis						
Sample ID	Trap ID	Collection day	Sample age	Wolf ID (video)	Total no. ind.	Marking behaiour	Morphology	Individual diagnostic traits	Genotype	Sex	Q_{wolf}	Y-haplotype	mt-haplotype	Total no. samplings	Sampling years
1	PO5	17/08/2013	5 days	αF8-MF	2	FLU + FM	wild type	long-haired tail	AP40	F	0.957	–	W14	4	2
2	LG1	08/03/2014	10 days	nd	1	FM	wild type	no	nd	F	–	–	–	–	–
3	PO5	16/03/2014	4.5 days	βM2-PS	2	FM + GSC	wild type	reduced black streaks on front legs, dark mark in zygomatic region	nd	M	–	–	–	–	–
4	PO9	12/03/2014	14 h	αM1-PS	4	FM	wild type	pale spots in supraorbital region, long-haired tail	AP37	M	0.987	H02	W14	8	3
5	PO5	30/03/2014	3 days	αM7-MF	4	FM	wild type	no	AP34	M	0.991	H01	W14	5	3
6	PO22	28/08/2014	14 h	βF5-PS	4	SQU + FM	anomalous	dark coat color, reduced white mask on right side	AP43	F	0.977	–	W14	9	3
7	PO22	28/08/2014	14 h	αM1-PS	4	STU + FM	wild type	pale spots in supraorbital region, long-haired tail	AP37	M	0.987	H02	W14	8	3
8	MF7	07/12/2014	12 h	nd	3	FM	wild type	no	MF01	F	0.872	–	W14	1	1
9	PO5	23/01/2015	10 days	αF1-PS	1	FM + GSC	anomalous	anomalous coat color, reduced white mask, pale neck, pendant ear	nd	–	–	–	–	–	–
10	MF4	14/03/2015	3.5 days	αM22-MF	2	FM + GSC + RLU + GSC	wild type	thin-pointed tail	MF03	M	0.988	H01	W14	8	2
11	MF4	14/03/2015	5.5 days	βM27-MF	1	RLU + GSC + FM + GSC	wild type	pale patch on the shoulders	MF04	M	0.944	H01	W14	3	3
12	PO22	15/03/2015	5 days	αF1-PS	3	FM	anomalous	anomalous coat colour, reduced white mask, pale neck, pendant ear	AP11	F	0.873	–	W14	15	6
13	PO24	28/03/2015	3 days	αM1-PS	1	FM + GSC	wild type	pale spots in supraorbital region, long-haired tail	AP37	M	0.987	H02	W14	8	3
14	MF7	21/03/2015	24 h	αM22-MF	2	RLU + FM + GSC	wild type	thin-pointed tail	MF03	M	0.988	H01	W14	8	2
15	MF7	21/03/2015	24 h	αF-MF	2	FM + FLU	wild type	no	MF05	F	0.959	–	–	1	1
16	ACN8	13/04/2015	5 h	αM15-CN	2	RLU + GSC + FM	anomalous	dark back, contrasting white mask, long-haired tail, spur on hind legs	AC55	M	0.973	H03	W14	3	2
17	PO24	14/06/2015	11 days	βM-PS	1	FM	anomalous	almost black coat color	AP51	M	0.895	H02	W14	2	2
18	ACN8	05/05/2015	9 days	αF18-CN	2	FM + GSC	wild type	thin tail, white anal patch	AC60	F	0.990	–	W14	2	2
19	PO9	04/08/2015	4 h	αF1-PS	4	FLU	anomalous	anomalous coat color, reduced white mask, pale neck, pendant ear	AP11	F	0.873	–	W14	15	6
20	PV2	20/05/2016	12.6 days	αM-VC	1	FM + GSC	wild type	no	nd	–	–	–	–	–	–
21	PO10	17/04/2016	4 days	αF6-PNE	2	FM + GSC	wild type	no black streaks on front legs, white socks, large white mask	nd	–	–	–	–	–	–
22	PO21	17/04/2016	1 day	αM-PN	2	FM	wild type	dark back, large neck, reduced black streaks on front legs	AP57	M	0.969	H01	W14	2	1
23	PO24	05/06/2016	2.5 days	αM-PS	1	RLU + FM + GSC	wild type	pale patch on the shoulders	MF04	M	0.944	H01	W14	3	3
24	PO5	29/01/2016	7.8 days	αM22-MF	2	FM + GSC	wild type	thin-pointed tail	MF03	M	0.988	H01	W14	8	2

Figure 1. Two images of defecating wolves extracted from videos recorded during remote camera trapping surveys in the Arezzo province (Italy, 2013–2016).

study area – which showed both morphological and genetic signatures of introgression and were members of a breeding pair. This evidence raises a major concern, as their successful breeding would lead to a further spread of canine genes in the population. Successful mating by wolf–dog hybrids in the wild was reported in central Italy (Caniglia et al. 2013) and had previously been hypothesized on the basis of the high proportion of backcrosses observed in other areas of Italy (Randi et al. 2014). Moreover, the spread of domestic genes into the Italian wolf gene pool is particularly worrying, given that this population is sharply differentiated genetically from any other wolf population due to its protracted isolation and past demographic declines (Montana et al. 2017).

As expected, the use of 'video-scats' was found to have a bias in favor of alpha individuals, owing to their stronger marking activity. Indeed, most of the sampled individuals (77%) were alpha mates. This represents a great advantage of our combined method, as they are typically the only individuals to breed in a pack (Mech and Boitani 2003). Hence, they genetically identify the social unit and, if introgressed, may transmit canine genes to the next generations. Despite the limited number of 'video-scats' collected and genotyped, we were able to sample the breeders of four different neighboring packs in our study area, thus assessing their morphological identity, breeding status and introgression level. Additionally, a non-negligible secondary advantage of obtaining the alpha pairs genotypes is that of speeding up both the process of pedigree reconstruction and the identification and removal of genotyping errors from the database (e.g. simplifying the unmasking of allelic dropout/false alleles in the offspring).

The main limitation of our approach was the low frequency of defecation events at CT sites. Although cameras were located at scent marking points along trails frequently used by wolves, the chance to film a wolf defecating was generally low (0.64 events over 100 trap days). It was, however, highly variable during the year, reaching a maximum in late summer (1.2–1.3 defecations over 100 trap days in August–September).

It should nonetheless be considered that the experimental design and placement of CT in our study area was focused on estimating pack density (Mattioli et al. unpubl.) in the framework of a monitoring program of the wolf population. The efficiency of our method was clearly affected by this limitation. In particular: 1) no lure

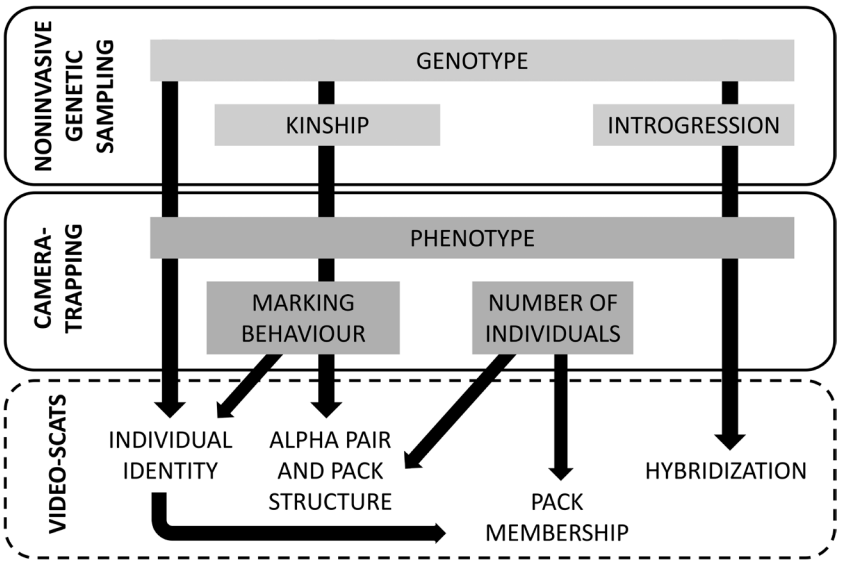

Figure 2. Additional information deriving from the integration of non-invasive genetic sampling and camera trapping for the monitoring of wolf populations. Genotyping allows for kinship analyses and the evaluation of introgression of domestic genes into the wolf gene pool; camera trapping provides information on phenotypic traits, marking behavior and pack size. Implemented together (i.e. analyzing 'video-scats'), the two approaches support one each other in individual recognition, pack assignment, identification of alpha pair and pack structure, and hybridization assessment.

was used to attract wolves to CT sites; 2) cameras were relatively far from each other in order to guarantee independent capture events; 3) the frequency of visits to CT sites was reduced in order to limit disturbance. This prevented us from prioritizing and optimizing the collection of 'video-scats', ultimately increasing the efforts necessary to obtain and successfully analyze each single 'video-scat'. On the other hand, in such a context, our method represented an easily applicable and almost costless way for gathering complementary information on individual wolves, supporting the identification of hybrid individuals and alpha pairs, and also helping in the reconstruction of pack membership and structure.

In conclusion, 'video-scats' represent a source of insightful information otherwise difficult to gather. They can be used in two main contexts: 1) within an already-existing monitoring program, where CT and NGS are already applied (like in our case); this method can be implemented virtually without any additional costs, but it will have some limitations and a reduced efficiency; 2) as an ad hoc study; this method can be significantly more efficient although it will necessarily be more expensive in terms of time and costs of equipment, personnel and laboratory analyses. Additionally, the method cannot be immediately applied to a completely unknown population, since it requires a preliminary identification of the scent marking sites used by packs and operators trained to handle video analysis.

In setting future studies based on 'video-scats', we thus suggest the following adjustments so as to maximize efficiency: 1) using a bait (possibly alien scats or urine) at camera-traps may increase the frequency of visits by wolves; 2) each camera trap should be ideally checked by operators once a week, in order to maximize the freshness of the scats collected (we observed a marked decline in the amplification success of fecal DNA collected around 10 days after defecation); 3) the best marking sites should be used, regardless of their proximity, also considering the possibility to set more cameras at a single site if large; 4) special attention should be paid to the quality of cameras and videos; 5) areas with a sufficient road network should be selected, so that CT sites can also be reached easily.

Our study ultimately showed that 'video-scats' analysis can be a useful and flexible tool to deepen our knowledge on the wolf packs inhabiting a given area, and can be especially effective for the management of admixed populations. We expect that this integrated approach, with possible adjustments, shall be useful for monitoring other species of elusive mammals in a non-invasive and almost costless fashion.

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