



Research Article

From predation to management: monitoring wolf distribution and understanding depredation patterns from attacks on livestock

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Abstract

The Italian wolf population, close to extinction in the mid-19th century, now counts about 1800 individuals. Its ongoing expansion raises social conflicts, especially in agricultural and semi-urbanized areas. Thus, monitoring wolf distribution, abundance and impact on the farming economy is a priority for conservation. We analysed canid DNA from 57 swabs from livestock kills, 13 faeces and 21 carcasses, to estimate the minimum number of individuals, their genetic variability and taxon (wolf, dog or hybrids), reconstruct the structure of local wolf packs, and describe the possible hunting patterns in a hitherto poorly investigated area of the Central Apennines. We genotyped, at the mitochondrial DNA control region and at 12 autosomal and four Y-linked microsatellites, 38 swab, three faecal and 19 muscular samples, corresponding to 42 individuals that Bayesian and Multivariate analyses assigned to 28 wolves, nine dogs and five admixed individuals. The minimum number of detected wolves ranged annually from three (2009) to 13 (2011), whereas parentage analyses identified at least three packs with a mean minimum home range of 60±48 km² and a mean pack size of 4.0±0.9 individuals. The identification of the genetic profiles of the animals involved in the predations revealed that livestock were killed by at least 13 wolves and four dogs, identifying cases of single-individual attacks and cases of cooperation of individual pairs. Integrating information from multigenerational pedigrees with predation patterns we could hypothesize that i) one pack increased livestock attacks after its disruption; ii) one pack showed a mother-offspring collaboration; iii) another pack started livestock predations after two unrelated individuals established a breeding pair. Our analyses of livestock predation events provided useful information on wolf population dynamics, that can be incorporated into local wolf management actions in areas where a regular monitoring is lacking and the predation risk is high.

Introduction

The Italian wolf population, which was close to extinction at the end of the Second World War, with approximately 100 surviving individuals (Zimen and Boitani, 1975), now counts more than 1800 wolves distributed in the whole Peninsula and part of the Alps (Galaverni et al., 2016), both in remote and nearly-urbanized areas (Caniglia et al., 2014), representing an unquestionable conservation success (Chapron et al., 2014). This ongoing expansion, on one side, arouses a deep interest for a flagship species that positively influences ecosystem equilibria (Ripple et al., 2014). However, on the other side, it is also exacerbating conflicts with human activities, especially in zones where breeders abandoned traditional husbandry practices (Dalmasso et al., 2012; Linnell and Boitani, 2012; Marino et al., 2016). In some areas of central-southern Italy, the wide diffusion of free-ranging dogs, whose predations on livestock can be erroneously attributed to wolves, is further reducing the levels of acceptance, though only a few studies to date have investigated the real proportions of predations performed by dogs (Caniglia et al., 2013; Imbert et al., 2016). Moreover, in other areas, such as Tuscany (Magrini, 2014; Marino et al., 2016), the human-wildlife relationship is worsened by the presence of wolf-dog hybrids (Bassi et al., 2017; Galaverni et al., 2017), according to the untested hypothesis that, being more confidential towards humans, hybrids might

be more dangerous for livestock (Caniglia et al., 2016; Bassi et al., 2017), and to the opinion that, not being pure wolves, they should not be ensured legal protection. For these reasons, livestock predations still represent one of the main issues for wolf conservation in Italy, since most of the known illegal killings occur in areas with high farm densities and high levels of damages, such as Tuscany and Piemonte, although without a perfect correlation (Boitani et al., 2010; Magrini, 2014; Galaverni et al., 2016; Marino et al., 2016). To mitigate these conflicts, a series of prevention measures (adequate animal husbandry compatible with the presence of predators, electric fences, guardian dogs) and compensation programs are regionally applied in Italy (Boitani et al., 2010; Dalmasso et al., 2012; Marino et al., 2016). Moreover, to favor the tolerance of local populations, some regional administrations generally compensate for canid damages, independently of whether wolves, dogs or wolf-dog hybrids were responsible for the attacks (Caniglia et al., 2013). However, compensation strategies revealed to be not always efficient because of their unsustainable long-term costs, and because they can indirectly encourage reporting false predations for compensations (Boitani et al., 2010). Moreover, heavy poaching and illegal persecutions persist in the whole Peninsula, also when compensations are ensured (Boitani et al., 2010; Dondina et al., 2015; Marino et al., 2016; Zingaro and Boitani, 2017), and lethal control is still believed by part of the stakeholders, by the public opinion and by the breeders to be a valid alternative tool for reducing damages (Linnell and Boitani, 2012; Kaltenborn and Brainerd, 2016). Nevertheless, a number

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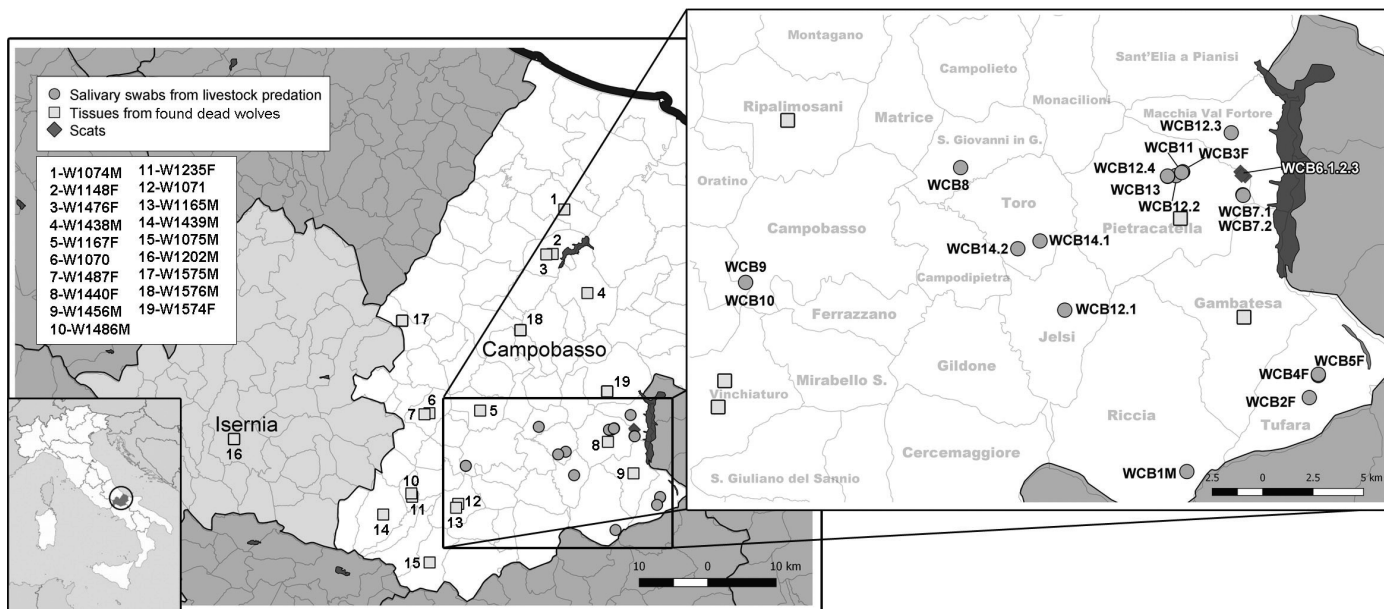


Figure 1 – Study area and different kinds of collected and analyzed samples. Circles indicate salivary swabs sampled from predated livestock heads, diamonds indicate scats and squares indicate muscular tissues sampled from found dead wolves.

of documented cases demonstrate that wolf killings seldom reduce the attacks, since mortality can weaken the pack structure or leave vacant territories that are soon filled by new breeding pairs or solitary inexperienced individuals that could further promote livestock predations (Wielgus and Peebles, 2014; Fernández-Gil et al., 2016; Imbert et al., 2016). Monitoring projects yielding reliable data about wolf abundance, pack structure and livestock predation patterns can provide basic information necessary to carry out effective management plans to reduce damages and associated costs, thus can mitigate conflicts in wildlife-people coexistence. Molecular tools have been successfully used to reconstruct livestock predation patterns and identify predators’ genotypes from salivary swabs collected on killed animals (Sundqvist et al., 2007; Harms et al., 2015) suggesting that, especially in high-risk contexts, integrating such data with information obtained from other non-invasive approaches, as snow-tracking, camera trapping and wolf-howling surveys (Galaverni et al., 2012; Caniglia et al., 2014; Canu et al., 2017; GranrothWilding et al., 2017; Suter et al., 2017) could greatly improve monitoring results and give valuable information on the relationship between wolf pack structure and livestock predation patterns.

In this work we preliminary monitored the number and structure of wolf packs living in the eastern Central Apennines (Campobasso province), where no systematic studies have been carried out to date, and limited ecological data are available only from local reports or indirect extrapolation (Galaverni et al., 2016). We analysed canid DNA extracted from faeces, wolf carcasses and salivary swabs obtained from predated livestock (sheep, goats) with the aim to: I) investigate the taxon (wolves, dogs or wolf-dog hybrids) of the individuals living in the study area, especially of those responsible for the livestock attacks; II) estimate the minimum number of individuals, their genetic variability and their putative pack structure; III) clarify, thanks to information available from pedigree reconstruction, the possible hunting patterns correlated to the structure of the packs and to the possible social role (e.g. breeders, juveniles or unrelated) of the individuals involved in the attacks.

Materials and methods

Study area

The study area mainly fell in the southern portion of the Campobasso Province (Molise Region) in the eastern side of the central Apennine ridge, with an average altitude of 576.7 m above sea level (Fig. 1), characterized by a temperate Mediterranean climate with an average temperature of 14.2 °C and a mean rainfall of 800 mm per year. The vegeta-

tion is mainly temperate and sub-Mediterranean with deciduous forests covering 14% of the Province and the artificial lake “Lago d’Occhito” (the lake is a Site of community Interest – SCI – and a Special Protection Area – SPA – of the Natura 2000 network). Several bird and mammal species live in the study area, where the wolf is the only large carnivore with a stable population. Historical records confirmed that in this area the wolf drastically declined at the beginning of the 1900s, almost reaching the extinction in the ‘70s (Zimen and Boitani, 1975), as in most of the Peninsula, mainly due to anthropic persecutions. Since the ‘80s the species reappeared, but data regarding its abundance and distribution are fragmented and inconsistent. The first signs of the return of the predator trace back to 1998, when a livestock kill was found with typical wolf bite patterns, and the presence of the species was confirmed in 2000 by the discovery of a dead wolf.

Natural habitats are fragmented by the widespread distribution of small villages and roads (about 77 inhabitants/km²), with livestock production representing the most important economic activity in the area. Sheep and goats are the most abundant livestock bred in the province (excluding poultry), with 53389 heads (18.3 heads/km² and 33.5 sheep farms/100 km²), followed by cattle (n=30710) and swine (n=13938; ISTAT 2010).

Sample collection

Muscular tissue samples were opportunistically collected from 21 putative wolves found dead in the study area from 2009 to 2013 (Tab. 1). For each sample, a fragment of about one cm³ of muscle was cut and stored in 50 ml of ethanol 95% at –20 °C.

A total of thirteen apparently fresh-looking faecal samples were found and collected in an area of 143.76 km² where ten fixed trails for a total of 9.43 km were selected (Fig. 1). Transects were checked twice a month from April 2009 to August 2013. To optimize the genotyping success we only collected samples recently deposited, well hydrated and without mildew, which were stored in 50 ml of ethanol 95% at –20 °C.

Salivary samples were collected from intra-vitam bite wounds on 57 animals killed or injured (41 sheep, nine goats, seven cattle) during 28 presumed canid kills from 24 farms between April 2010 to May 2013. Samples were collected within 36 hours after each attack using a sterile cotton swab per bite rubbed on the skin and hairs around the bite, or cutting fragments of ca. 3×3 cm of skin near the bite. The cotton swabs were air-dried and stored in sterile tubes, whereas tissues were stored in 50 ml of 95% ethanol. When more than one wound was present on a single killed animal, samples from each lesion were collected and stored separately (Tab. 1). All salivary samples were collected

Table 1 – Field information (sample type, sampling year, cause of death or farm) and molecular identifications (taxon, gender, Structure results, Y-STR and mtDNA haplotypes, genotype probability, pack number) of samples collected in the study area. **ID-S:** sample identification; **ID-G:** genotype identification; **q_w, CI:** *q_w*, *CI*; assignment value to the wolf group and respective confidence interval obtained by Structure; **YH:** Y haplotype identification as named in Randi et al. (2014); **mtDNA-H:** mtDNA haplotype identification as named in Randi et al. (2000) or GeneBank accession number; **GP:** genotype probability (of two different individuals of sharing by chance the same genotype) cumulative product for increasing locus combinations; *nd:* not detected; *na:* not available; *M:* male; *F:* female.

ID-S	Sample type	Locality	Sampling year	Cause of death/Farm	Ancestry population	Gender	ID-G	q _w	CI	YH	mtDNA-H	GP	Pack ID
W1055	Muscular tissue	Macchia Valfortore	2009	unknown	nd		nd						
W1070	Muscular tissue	Castropignano	2009	road-kill	W-D admixed	F	H1070F	0.976	0.819–1.000	na	W14	6 × 10 ⁻¹¹	
W1071	Muscular tissue	Vinchiaturo	2009	road-kill	wolf	F	W1071F	0.998	0.992–1.000	na	W14	1.5 × 10 ⁻⁹	1>3
W1074	Muscular tissue	Palata	2010	road-kill	wolf	M	W1074M	0.998	0.993–1.000	YH17	W14	2.3 × 10 ⁻⁸	1
W1075	Muscular tissue	Guardiaregia	2010	intraspecific fight	wolf	M	W1075M	0.994	0.966–1.000	YH17	W14	2 × 10 ⁻⁹	
W1148	Muscular tissue	Guardiafiera	2011	poaching	wolf	F	W1148F	0.997	0.986–1.000	na	W14	1.1 × 10 ⁻⁷	2
W1165	Muscular tissue	Vinchiaturo	2011	road-kill	wolf	M	W1165M	0.998	0.994–1.000	nd	nd	3.8 × 10 ⁻⁸	
W1166	Muscular tissue	Sant'Elia a Pianisi	2011	unknown	nd		nd						
W1167	Muscular tissue	Ripalimosani	2011	road-kill	W-D admixed	F	H1167F	0.914	0.715–1.000	na	W14	4.4 × 10 ⁻⁸	
W1202	Muscular tissue	Isernia	2011	road-kill	wolf	M	W1202M	0.997	0.988–1.000	YH26	W14	1.1 × 10 ⁻⁷	2
W1235	Muscular tissue	Colle d'Anchise	2012	poaching	wolf	F	W1235F	0.998	0.993–1.000	na	W14	5.9 × 10 ⁻⁸	
W1438	Muscular tissue	Casacalenda	2012	scabies	wolf	M	W1438M	0.998	0.994–1.000	YH17	W14	4.7 × 10 ⁻⁷	
W1439	Muscular tissue	Bojano	2012	road-kill; prob poisoned	wolf	M	W1439M	0.997	0.989–1.000	YH17	W14	7 × 10 ⁻¹⁰	1
W1440	Muscular tissue	Pietracatella	2012	road-kill; prob poisoned	W-D admixed	F	H1440F	0.805	0.608–0.961	na	W14	4.9 × 10 ⁻⁸	
W1456	Muscular tissue	Gambatesa	2012	road-kill	W-D admixed	M	H1456M	0.993	0.958–1.000	YH05	W14	9.2 × 10 ⁻⁸	
W1476	Muscular tissue	Guardiafiera	2013	road-kill	wolf	F	W1476F	0.997	0.990–1.000	na	W14	1.5 × 10 ⁻¹¹	
W1486	Muscular tissue	Colle d'Anchise	2013	road-kill	wolf	M	W1486M	0.998	0.993–1.000	YH17	W14	2.6 × 10 ⁻¹⁰	1
W1487	Muscular tissue	Castropignano	2013	road-kill	wolf	F	W1487F	0.998	0.993–1.000	na	W14	6.3 × 10 ⁻⁹	
W1574	Muscular tissue	Sant'Elia a Pianisi	2011	road-kill	W-D admixed	F	H1574F	0.858	0.681–1.000	na	W14	2.9 × 10 ⁻⁸	
W1575	Muscular tissue	Salcito	2011	road-kill	wolf	M	W1575M	0.998	0.994–1.000	YH26	W14	6.5 × 10 ⁻¹⁰	3
W1576	Muscular tissue	Castellino sul Biferno	2012	intraspecific fight	wolf	M	W1576M	0.997	0.986–1.000	YH17	W14	3.3 × 10 ⁻⁹	1
CB002	Salivary	Riccìa	2010	A	nd		nd						
CB003	Salivary	Riccìa	2010	A	wolf	M	WCB1M	0.996	0.984–1.000	YH17	nd	5.4 × 10 ⁻⁷	2
CB004B	Salivary	Sepino	2010	B	dog	M	DCB1M	0.013	0.000–0.097	YH06	nd	4.2 × 10 ⁻¹¹	
CB005	Salivary	Petrella Tifermina	2010	C	dog	F	DCB2F	0.002	0.000–0.009	na	D5; D6	9.8 × 10 ⁻¹¹	
CB006B	Salivary	Tufara	2010	D	wolf	F	WCB2F	0.993	0.960–1.000	na	nd	3.2 × 10 ⁻⁸	2
CB007A	Salivary	Castellino Nuovo	2010	E	nd		nd						
CB008A	Salivary	Sant'Elia a Pianisi	2010	F	nd		nd						
CB009A	Salivary	Pietracatella	2010	G	wolf	F	WCB3F	0.998	0.991–1.000	na	nd	6.3 × 10 ⁻⁸	2
CB009C	Salivary	Pietracatella	2010	G	nd		nd						
CB010	Salivary	Gambatesa	2010	Y	nd		nd						
CB011F	Salivary	Tufara	2010	H	wolf	F	WCB4F	0.997	0.985–1.000	na	nd	1.3 × 10 ⁻⁸	2
CB012	Salivary	Tufara	2010	H	nd		nd						
CB013A	Salivary	Tufara	2010	H	nd		nd						
CB014A	Salivary	Tufara	2010	H	wolf	F	WCB5F	0.997	0.989–1.000	na	nd	1.6 × 10 ⁻⁷	2
CB015A	Salivary	Tufara	2010	H	wolf	F	WCB5F						2
CB016A	Salivary	Tufara	2010	H	wolf	F	WCB5F						2
CB017A	Salivary	Tufara	2010	H	wolf	F	WCB5F						2
CB078A	Salivary	Campolieto	2010	I	nd		nd						
CB079A	Salivary	Riccìa	2010	L	dog	nd	DCB3	0.013	0.000–0.097		AF531680	2.8 × 10 ⁻¹¹	
CB080B	Salivary	Riccìa	2010	L	dog	nd	DCB3						
CB081B	Salivary	Jelsi	2010	M	wolf	nd	WCB12	0.996	0.978–1.000		W14	4.3 × 10 ⁻⁸	2
CB082B	Salivary	Pietracatella	2010	N	wolf	nd	WCB7	0.998	0.992–1.000		W14	4.6 × 10 ⁻⁸	2
CB083A	Salivary	Pietracatella	2010	N	wolf	nd	WCB7						2
CB084B	Salivary	Campobasso	2011	O	dog	nd	DCB4	0.002	0.000–0.009		JF342882	7.3 × 10 ⁻⁶	
CB085B	Salivary	Campolieto	2011	I	nd		nd						
CB086A	Salivary	San Giovanni in Galdo	2011	P	wolf	nd	WCB8	0.990	0.09–1.000		W14	9 × 10 ⁻⁹	3
CB088B	Salivary	Castropignano	2011	Q	nd		nd						
CB089A	Salivary	Castropignano	2011	Q	nd		nd						
CB090	Salivary	Campolieto	2011	I	dog	nd	DCB6	0.002	0.000–0.008		D14	5.5 × 10 ⁻⁹	
CB091A	Salivary	Busso	2011	R	wolf	nd	WCB10	0.998	0.992–1.000		W14	5 × 10 ⁻⁵	3
CB092B	Salivary	Busso	2011	R	wolf	nd	WCB9	0.998	0.992–1.000		W14	2.8 × 10 ⁻⁹	
CB093A	Salivary	Busso	2011	R	wolf	nd	WCB9						
CB094B	Salivary	Busso	2011	R	wolf	nd	WCB9						
CB095A	Salivary	Pietracatella	2011	G	nd		nd						
CB096A	Salivary	Pietracatella	2011	G	nd		nd						
CB097B	Salivary	Pietracatella	2011	G	dog	nd	DCB7	0.003	0.000–0.008		nd	4.1 × 10 ⁻⁷	
CB098B	Salivary	Pietracatella	2011	G	wolf	nd	WCB11	0.990	0.989–1.000		W14	3 × 10 ⁻⁶	
CB099	Salivary	Baranello	2011	T	nd		nd						
CB100	Salivary	Baranello	2011	T	dog	nd	DCB8	0.004	0.000–0.019		D5; D6	6.7 × 10 ⁻⁹	
CB101	Salivary	Baranello	2011	T	dog	nd	DCB9	0.004	0.000–0.016		D13; D15	8.9 × 10 ⁻⁹	
CB102	Salivary	Baranello	2011	T	dog	nd	DCB9						
CB104A	Salivary	Macchia Valfortore	2011	Z	wolf	nd	WCB12						2
CB105A	Salivary	Pietracatella	2011	PB	wolf	nd	WCB12						2

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Table 1 – (continued) Field information (sample type, sampling year, cause of death or farm) and molecular identifications (taxon, gender, Structure results, Y-STR and mtDNA haplotypes, genotype probability, pack number) of samples collected in the study area. **ID-S**: sample identification; **ID-G**: genotype identification; **q_w**, **CI**: q_i assignment value to the wolf group and respective confidence interval obtained by Structure; **YH**: Y haplotype identification as named in Randi et al. (2014); **mtDNA-H**: mtDNA haplotype identification as named in Randi et al. (2000) or GeneBank accession number; **GP**: genotype probability (of two different individuals of sharing by chance the same genotype) cumulative product for increasing locus combinations; *nd*: not detected; *na*: not available; *M*: male; *F*: female.

ID-S	Sample type	Locality	Sampling year	Cause of death/Farm	Ancestry population	Gender	ID-G	q _w	CI	YH	mtDNA-H	GP	Pack ID
CB105E	Salivary	Pietracatella	2011	PB	wolf	nd	WCB13	0.998	0.994–1.000		W14	1.2 × 10 ⁻⁷	2
CB106A	Salivary	Mirabello	2011	ZL	dog	nd	DCB11	0.002	0.000–0.009		D1	1.1 × 10 ⁻⁹	
CB107A	Salivary	Mirabello	2011	ZL	dog	nd	DCB11						
CB108A	Salivary	Mirabello	2011	ZL	dog	nd	DCB11						
CB109A	Salivary	Mirabello	2011	ZL	dog	nd	DCB11						
CB110A	Salivary	Mirabello	2011	ZL	dog	nd	DCB11						
CB111B	Salivary	Mirabello	2012	ZL	dog	nd	DCB11						
CB112A	Salivary	Toro	2011	MG	wolf	nd	WCB14	0.990	0.904–1.000		W14	2 × 10 ⁻⁹	1
CB116	Salivary	Toro	2012	ND	wolf	nd	WCB14						1
CB124	Salivary	Gildone	2013	DB	nd	nd							
CB125	Salivary	Gildone	2013	DB	nd	nd							
CB126	Salivary	Gildone	2013	DB	nd	nd							
CB127	Salivary	Gildone	2013	DB	nd	nd							
CB128	Salivary	Gildone	2014	DB	nd	nd							
CB069	Scat	Pietracatella	2009		nd	nd							
CB070	Scat	Pietracatella	2009		nd	nd							
CB071	Scat	Pietracatella	2009		nd	nd							
CB072	Scat	Pietracatella	2009		wolf	nd	WCB6	0.990	0.933–1.000		nd	1.2 × 10 ⁻⁷	3
CB073	Scat	Pietracatella	2009		wolf	nd	WCB6						3
CB074	Scat	Pietracatella	2009		nd	nd							
CB075	Scat	Pietracatella	2009		wolf	nd	WCB6						3
CB076	Scat	Pietracatella	2009		nd	nd							
CB077	Scat	Pietracatella	2009		nd	nd							
CB117	Scat	Pietracatella	2013		nd	nd							
CB118	Scat	Pietracatella	2013		nd	nd							
CB129	Scat	Gildone	2013		nd	nd							
CB149	Scat	Macchia Valfortore	2013		nd	nd							

by a veterinary expert who carried out a preliminary diagnosis describing predation patterns, evaluating the presence of sub-dermal haemorrhages behind the wounds to assert the putative predators, presumed time and the most likely causes of death or evidences of post-mortem consumption by scavengers (Caniglia et al., 2013). In cases of injured animals, all the necessary procedures were taken to minimize the stress and disturbance, always respecting animal welfare procedures. No animal was hurt nor sacrificed for the purposes of this study. The coordinates of every sample were recorded on a 1:25000 topographic map or by global positioning system devices and digitalized on ARCGIS v.10.0 (ESRI, Redlands, California).

DNA extraction and marker amplification

Total DNA was extracted from salivary swabs using the Zymo Research Quick gDNA Miniprep Kit (Zymo Research Corporation, USA), adding a pre-digestion step a 56 °C for 45’ in a lysis buffer (WCLB, proteinase k, SDS 10%), whereas DNA from tissue and scat samples was extracted using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer’s instructions.

Each DNA sample was amplified by Polymerase Chain Reaction (PCR) and genotyped, through a multiple-tube approach (Taberlet, 1996; Caniglia et al., 2014), at 12 canine unlinked autosomal microsatellites (FH2004, FH2079, FH2088, FH2096, FH2137, CPH2, CPH4, CPH5, CPH8, CPH12, C09.250, C09.253), which have been already successfully used for individual identifications in long-term non-invasive monitoring projects of the Italian wolf population (Caniglia et al., 2014; probability of identity computed in reference wolf population, among unrelated individuals PID=9.6 × 10⁻⁹ and expected full-sibling PIDsibs=3.5 × 10⁻⁴), for forensic applications (Caniglia et al., 2016) and for the discrimination between wolves, dogs and their first two-three generation hybrids through Bayesian assignment procedures (Caniglia et al., 2014; Randi et al., 2014). DNA Samples were also sexed by a PCR-RFLP assay of the zinc-finger protein gene ZFX/ZFY (Lucchini et al., 2002) and paternal haplotypes in male individuals were identified by the amplification of four Y-linked

STRs (MS34A, MS34B, MSY41A, MSY41B; Sundqvist et al., 2001). Autosomal and Y-linked STRs were amplified in seven multiplexed reactions using the QIAGEN Multiplex PCR kit (Qiagen Inc., Hilden, Germany).

Each DNA was also PCR-amplified in 10 µL of final volume, including 2 µL of DNA and 0.3 pmol of primers WDLOOP and WDloopH254 (Caniglia et al., 2013) at the first 250 bp of the mtDNA control-region, containing diagnostic mutations for the Italian wolf population (haplotypes W14 and W16; Randi et al., 2000; Montana et al., 2017). PCR products were sequenced in both directions, after purification with exonuclease/shrimp alkaline phosphatase (Exo-Sap; Amersham), using the ABI Big Dye Terminator kit v.3.1.

PCR products were analyzed in an ABI 3130XL automated sequencer. Sequences were visually edited using the ABI software SEQSCAPE v.2.5 and aligned with BioEDIT (Hall, 1999). Identical haplotypes were matched using DNASP v.5.0 (Librado and Rozas, 2009) and compared with sequences available from GenBank using BLAST (Altschul et al., 1990). The allele sizes of STR loci were estimated using the ABI ROX 350 size standard and the ABI software GENEMAPPER v.4.0. We run GENEMAPPER following all the recommendations of the Process Quality Value Tests for basic troubleshooting about stutters, quality, weight and width of allele peaks and applying Bin Alleles defined using only good-quality canid DNA samples. For further details on PCR conditions and thermal profiles see Caniglia et al. (2013).

Extraction, amplification and post-amplification procedures of salivary, faecal and muscular DNA were carried out in separate rooms reserved to low-template DNA samples, adding a blank control (no biological material) during DNA extraction, and a blank (no DNA) and a positive (known wolf-DNA sample) controls during DNA amplification.

Error rate and reliability analysis

Following the multi-tube protocol described in Caniglia et al. (2013), non-invasive DNAs (salivary and faeces) were amplified at the autosomal STRs from four to eight independent times per locus.

After the first four replicates at the 12 autosomal STR loci, samples showing $\leq 50\%$ positive PCR (PCR+) were discarded. A reliability analysis was performed by the software RELIOTYPE (Miller et al., 2002) on samples showing $>50\%$ PCR+, and unreliable loci (at threshold $R < 0.95$) were additionally replicated another four times. Only samples reliably typed at all loci ($R \geq 0.95$) were definitively accepted, molecularly sexed and genotyped at uniparental markers (mtDNA control region, Y-linked STRs).

Consensus genotypes were reconstructed from the 4–8 replicates using GIMLET v.1.3.3 (Valière, 2002), accepting heterozygotes only if both alleles were seen in at least two replicates, and homozygotes only if a single allele was seen in at least four replicates. GIMLET was also used, following Pompanon et al. (2005), to estimate PCR success rate (the number of successful PCRs divided by the total number of PCR runs across samples), allelic drop-out (ADO) and false alleles (FA) and to match the detected genotypes to each other and with the ISPRa *Canis* database. The cumulative probability product for increasing locus combinations of two wolves sharing by chance the same genotype was calculated using GENALEX v.6.5 (Peakall and Smouse, 2012).

Taxon identification

Two different methodologies were used to assign the 12-STR multi-locus genotypes to their taxon (wolf, dog or admixed): (i) a principal coordinate analysis (PCoA) implemented in GENALEX 6.5 (Peakall and Smouse, 2012); (ii) a Bayesian clustering procedure implemented in the program STRUCTURE v.2.3.4 (Pritchard et al., 2000), which estimates the admixture proportion of each individual genotype independently of any prior non-genetic information.

As reference populations we selected 190 unrelated wild individuals belonging to the Italian wolf population (112 males and 78 females, which can be considered to be representative of the entire population since they were sampled across the whole species' distribution range in Italy and not biased by any possible level of genetic substructure) and 89 wolf-sized dogs (40 males and 49 females) living in rural areas, from the ISPRa *Canis* database. All the selected wolves showed the typical Italian wolf coat color pattern and neither morphologically nor genetically detectable signs of hybridization (Randi et al., 2014; Galaverni et al., 2017).

In order to avoid biased assignment rates we singularly ran each unknown genotype parallelizing several STRUCTURE runs with identical setting using ParallelStructure (Besnier and Glover, 2013) an R package implementing STRUCTURE. We ran three repetitions of 5×10^5 iterations following a burn-in period of 5×10^4 iterations using the Admixture and independent allele frequencies (F) models (Falush et al., 2003) with $K=2$ (corresponding to the optimal number of genetic clusters in the dataset used, see Results). We assigned the unknown individual genotypes to the Italian wolf or dog clusters at threshold $q_i \geq 0.990$ (q_i =individual proportion of membership), or identified them as admixed if their q_i values were ≤ 0.989 due to dog private alleles or to alleles with a higher frequency in dogs.

Assignments were integrated with the information derived from the uniparental (mtDNA, four Y-linked STRs) markers, which were used to confirm the taxon identification or, in case of admixed individuals, to provide the directionality of the hybridization (Caniglia et al., 2014; Randi et al., 2014).

Variability and Relatedness analysis

Based on the assignment tests, we grouped the detected genotypes as wolves, dogs or admixed. Genetic variability indexes per group (allele frequency by locus and group, observed (H_o) and expected unbiased (uHE) heterozygosity, mean number of different (N_a) and effective (N_e) alleles and number of private alleles (PA) were estimated using GENALEX (Peakall and Smouse, 2012) and compared to those of the wolf and dog reference populations. We calculated the polymorphic information content (PIC) using CERVUS v.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 v.4.05 (Belkhir et al., 1996–2004). We assessed

F_{IS} significance using 10000 random permutations of alleles in each population.

We reconstructed the genealogy of the genetically identified wolves using a maximum likelihood approach implemented in COLONY v.2.0 (Wang and Santure, 2009). We ran COLONY with all the wolves as candidate parents, allele frequencies estimated from the whole reference wolf population, PCR error rates estimated by GIMLET, and probability of including fathers and mothers in the candidate parental pair of 0.5. We then verified genealogies using the approach based on the Mendelian inheritance of the alleles implemented in GIMLET, accepting only parent-offspring combinations of individuals contemporarily sampled but with at most one/twenty fourth allele incompatibilities, and father-son combinations with no incongruities at Y-STR haplotypes.

Sampling locations of the individuals associated in the pedigrees were used in ARCGIS v.10.0 to calculate the approximate home ranges of the hypothetical packs using a Minimum Convex Polygon approach.

Results

Genetic identifications, genotyping rate, individual assignment

We collected a total of 70 non-invasive samples (57 from depredation sites inspections and 13 from scat surveys) and 21 invasive samples from putative wolves found dead (Tab. 1).

The first step of the multi-tube protocol led us to discard 24 non-invasive samples (34.3%) that showed $\text{PCR}+ \leq 0.50$. The other 46 non-invasive samples (65.7%) successfully passed the first step, showing $\text{PCR}+ > 0.50$. Eighteen of them (26%) had reliability scores $R \geq 0.95$ and were directly accepted. The other 28 non-invasive samples with $R < 0.95$ were further amplified four times at unreliable loci, after which only five of them were discarded, while the other 23 reached the threshold $R > 0.95$ and were definitively accepted. Thus, 41 (59%) non-invasive samples (38 predation salivary swabs and three faecal samples) were reliably genotyped at the 12 autosomal STRs. Regrouping procedures and error rate analysis indicated these samples corresponded to 23 distinct genotypes, showing an average number of positive amplifications per locus of 0.71 (ranging from 0.87 to 0.40), and average error rates $\text{ADO}=0.24$ ($\text{SD}=0.10$) and $\text{FA}=0.05$ ($\text{SD}=0.05$).

The 21 muscular samples produced 19 (90.5%) reliable and distinct genotypes, never sampled before, showing no evidence of ADO or FA errors.

The 60 complete genotypes were assigned to 42 distinct individuals, 14 females, 12 males and 16 with uncertain sex (whose DNA quality did not allow to complete their gender information), which did not match to any genotype of the ISPRa *Canis* database (Tab. 1).

Moreover, due to random DNA degradation, only 11 of the 12 detected males produced reliable Y haplotypes and 33 of the 42 distinct genotypes (15 non-invasive and in 18 invasive samples) yielded reliable mtDNA sequences (Tab. 1). After matching all identical sequences in DNASP and blasting them against GenBank (pairwise identity $>99.4\%$), we identified one wolf (W14; Randi et al., 2000) and six dog (D1, D5/6, D14, D13/15; Randi et al., 2000; A11-AF531680, A28-JF342882) unique mtDNA haplotypes.

The PCoA split wolf and dog reference individuals into two clearly separate clusters along the two principal axes (PC-I and PC-II), which cumulatively explain 30.8% of the total genetic diversity, with nine of the genotypes detected in the study area plotted among dogs and other 33 within wolves (Fig. 2B).

At $K=2$, corresponding to the optimal number of genetic clusters (likelihood mean \ln value = -7998.2), all reference dogs were assigned to a same cluster with average $Q_d=0.997$ (CI: 0.989–1.000) and all reference wolves were assigned to the other cluster with average $Q_w=0.997$ (CI: 0.989–1.000; Fig. 2A). Individual q_i ranged between 0.990 and 0.999 in dogs and between 0.992 and 0.999 in wolves. Among the 42 genotypes detected in the study area, 29 were assigned to the wolf cluster (q_w ranged between 0.990 and 0.998), nine to the dog cluster (q_d from 0.987 to 0.998), while four resulted admixed at the nuclear DNA ($q_w \leq 0.989$) showing a q_w from 0.805 to 0.976 due to the presence of private dog alleles (Tab. S1). Twenty-two of the individuals assigned

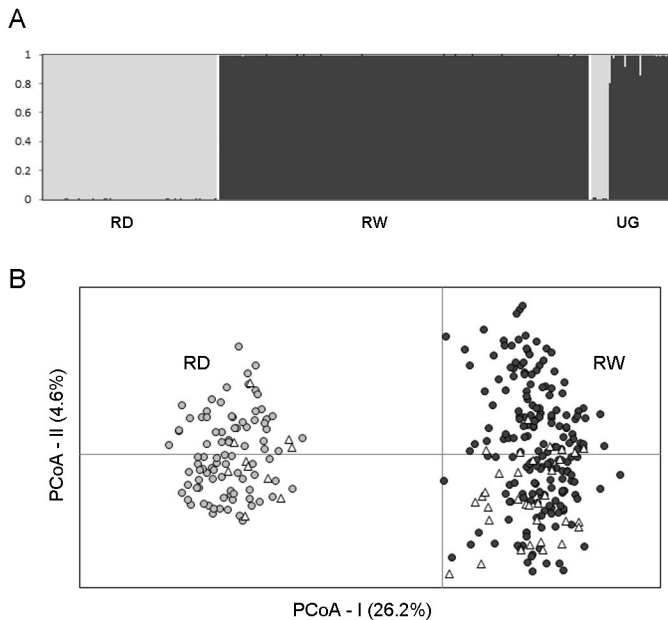


Figure 2 – A) Bayesian analysis obtained by Structure using admixture models and $K=2$. Each individual is represented by a vertical bar fragmented in K sections of different length, according to their membership proportion in the two inferred genetic clusters: the light grey bars represent the dog component and the dark grey ones the wolf component. RW=reference wolves, RD=reference dogs, UG=unknown genotypes from the study area. B) Principal Coordinate Analysis performed by GenAlEx. The two principal axes (PC-I and PC-II) cumulatively explain 30.8% of the total genetic diversity. Light grey dots represent the Italian dog reference (RD), dark grey dots the Italian wolf reference (RW) and white triangles the unknown genotypes detected in the study area.

to the wolf cluster (seven did not produce reliable mtDNA sequences) and the four admixed individuals showed the diagnostic W14 Italian mtDNA haplotype, whereas seven of the individuals assigned to the dog cluster (two did not produce reliable mtDNA sequences) showed dog mtDNA haplotypes (Tab. 1). Among the males assigned to the wolf cluster ($N=11$; $q_w \geq 0.990$) one did not amplify at the Y-STRs, nine showed the two private Italian Y-chromosome haplotypes (YH17 and YH26; Randi et al., 2014) and one showed a dog Y-chromosome haplotype (YH05; Randi et al., 2014), representing a case of Y-chromosome introgression thus it was subsequently considered as an admixed individual. Only one male assigned to the dog cluster ($q_d \geq 0.987$) produced a reliable Y-haplotype corresponding to the dog YH06 haplotype (Randi et al., 2014).

Genetic variability analysis

All the 12 autosomal microsatellites were polymorphic in the 28 pure wolves sampled in the study area. The five admixed individuals were excluded from these analyses to avoid the risk that alleles from dogs inflated estimates of genetic variability in the wolf population. Overall wolves showed from three to 12 alleles, with an average observed number of alleles per locus which significantly differed ($p=0.024$; F-test) between wolves in the study area ($N_a=3.83 \pm 0.34$) and reference wolves ($N_a=5.25 \pm 0.71$). However, wolves in the study area showed intermediate but not significantly different ($p>0.66$; F-tests) values of heterozygosity ($H_o=0.53$, $uH_e=0.53$, $PIC=0.47$) compared to reference wolves ($H_o=0.54$, $uH_e=0.57$, $PIC=0.52$).

Both wolves in the study area and reference wolves did not accord to Hardy–Weinberg expectations ($F_{IS}=-0.088$, $p=0.012$; $F_{IS}=-0.035$, $p=0.004$; Tab. S1).

Possible livestock predation patterns

Between 2010 and 2013, a total of 57 domestic animals were preyed upon at 24 farms during 28 attacks, with an average number of killed or injured animals per attack of 1.9 (ranging from one to six individuals; Tab. 2).

For 12 animals killed at six farms (E, DB, F, I, Q, Y) during seven attacks, it was impossible to identify neither the taxon nor the genetic

profile of the predator, even if the veterinary diagnosis suggested wolf-like predation patterns. Genetic analyses revealed that 14 attacks were performed by 13 wolves, involving 29 animals from 12 different farms, in accordance with veterinary reports (Tab. 2). In three occasions wolves attacked in free pastures without active electric fences, whereas in the other 11 cases livestock were enclosed in external corrals. All the wolf attacks were nocturnal or crepuscular and mostly occurred from April to August. Five farms were apparently attacked by solitary wolves (WCB1, WCB2, WCB7, WCB8 and WCB11), two farms by two wolves (WCB9 and WCB10 in farm R; WCB12 and WCB13 in farm PB), each killing one domestic animal during the same attack, and other two farms were attacked by two other wolves (WCB4 and WCB5 in farm H; WCB3 and WCB11 in farm G) but during different occasions (Tab. 2). Interestingly, in farm G, after an attack (attack number 21; Tab. 2) we identified the genetic profiles of a wolf (WCB11) and of a dog (DCB7), but this latter likely contaminated the prey post-mortem while scavenging on it. We detected a case in which one wolf (WCB5F) killed four sheep during the second attack in farm H, a case in which one wolf (WCB9) killed three sheep (farm R) and a case in which one wolf (WCB7) killed two sheep (farm N; Tab. 2).

Genetic investigations also revealed that eight dogs were responsible for the other seven attacks, preying on 16 animals. In one case (Farm ZL), the veterinary necropsy findings were inconsistent with genetic results since they identified wolf predation patterns on the carcasses of the six goats killed, although they all showed genetic traces of a single dog (DCB11) that could have acted as a scavenger, whereas no wolf DNA was identified by our analyses. Another necropsy (Farm B) revealed that one of the cattle on which we identified a dog genotype had died for an intestinal obstruction and was only consumed post-mortem. In two other attacks (farm C and farm I) veterinary diagnosis suggested post-mortem injuries from which genetic analyses identified the profiles of other two dogs (attacks number three and 19; Tab. 2). The other actual dog attacks were confirmed by the veterinary reports based on size and spacing of bite wounds on the prey and killing behavioural patterns. These attacks involved at least four dogs: two of them acted alone, whereas the other two cooperated in killing four cattle during the same attack in farm T (Tab. 2).

Reconstructions of putative wolf packs

Interpolating results from salivary, scat, and muscular DNA genotyping, we identified a minimum of three in 2009, nine in 2010, 13 in 2011, seven in 2012 and three in 2013 wild canids (including both wolves and admixed individuals). All these data were geographically mapped and used in parentage analyses among genotypes in COLONY. Results suggested the presence of at least three main clusters with pairs of genotypes having probability $p>0.950$ to be parents of one or more offspring and occupying an average home range of $60 \pm 48 \text{ km}^2$, for which we reconstructed hypothetical familial pedigrees (Fig. 3).

The first pack (Pack 1, Fig. 3) was located in a territory of about 130 km^2 in the central part of the study area, where it might have been present since 2009. Individuals WCB14 and W1074M (found dead in February 2012 killed by a car) showed a likelihood value of 0.999 to be putative parents of genotypes WCB12, W1071F (road-killed in 2009), W1202 (road-killed in 2011), W1439M and W1576M (died in 2012 for a car collision and for an intraspecific aggression, respectively).

The second hypothetical pack (Pack 2, Fig. 3) occupied a home range of 156 km^2 in the southern east part of the study area and was likely derived from an incestuous mating of individuals WCB12 and W1202M, brother and sister from Pack 1, which showed a likelihood score 0.999 to be the putative parents of other eight genotypes.

However, temporal patterns in sample collection, as well as wolf-howling and camera-trap surveys (D’Amico, 2014), suggested that these individuals could be more likely split into two different but related groups, occupying two adjacent territories separated by a highway: a southern group A (c. 30 km^2) including four genotypes (WCB1M, WCB2F, WCB4F, WCB5F) and a northern group B (c. 27 km^2) including other four (W1148F, WCB3F, WCB7, WCB13) genotypes (Fig. 3). This hypothesis seems well supported also by clear different livestock

Table 2 – Description of the farms that experienced livestock attacks. **ID-A:** attack identification number; **Ps:** prey species: C, cattle; G, goat; S, sheep; **N:** number of bred animals; **P:** number of animals killed or injured in the attack corresponding to the number of collected samples; **G:** number of genotyped forensic samples; **ID-G (Pack):** genotype (and pack) identification; **S:** taxon of the predators identified from genetic analyses; **VD:** veterinary diagnosis describing if livestock heads were intra-vitam killed by wolves or dogs, or post-mortem scavenged; **SD:** sampling date: dd/mm/yy; **Hr:** estimated time interval at which attacks occurred; **HE/ND:** height of the enclosure (in meters) / number of dogs owned by each farmer; *nd:* not detected.

ID-A	Farm	Locality	Ps	N	P	G	ID-G (Pack)	S	VD	SD	Hr	HE/ND
1	A	Riccia	S	15	2	1	CB1M(2)	wolf	W	27/04/2010	20.00–7.00	1/0
2	B	Sepino	C	3	1	1	DCB1M	dog	scavenged	27/04/2010	20.00–8.00	0/2
3	C	Petrella Tifernina	S	130	1	1	DCB2F	dog	Fox, scavenged by dog	10/05/2010	17.00	2/2
4	D	Tufara	S	15	1	1	WCB2F(2)	wolf	W	12/05/2010	19.30–8.00	1.5/0
5	E	Castellino Nuovo	C	6	1	1	nd	nd	W	2/06/2010	21.00–8.00	2.2/5
6	F	Sant’Elia a Pianisi	S	15	1	0	nd	nd	W	17/06/2010	20.00–7.00	2/3
7	G	Pietracatella	S	50	2	1	WCB3F(2)	wolf	W	20/07/2010	20.00–6.30	1.5/1
8	Y	Gambatesa	S	20	1	0	nd	nd	W	26/07/2010	7.00	1.8/0
9	H	Tufara	S	20	3	1	WCB4F(2)	wolf	W	31/07/2010	20.00–7.00	1.8/0
10	H	Tufara	S	20	4	4	WCB5F(2)	wolf	W	17/08/2010	3.00–6.00	1.8/0
11	I	Campolieto	S	10	1	0	nd	nd	D	24/09/2010	18.30	1.5/0
12	L	Riccia	C	70	2	2	DCB3	dog	D	27/09/2010	7.00	0/1
13	M	Jelsi	S	20	1	1	WCB12(2)	wolf	W	29/11/2010	20.00–7.00	1.8/1
14	N	Pietracatella	S	20	2	2	WCB7(2)	wolf	W	24/12/2010	21.00–7.00	1.8/2
15	O	Campobasso	G	10	1	1	DCB4	dog	D	15/02/2011	18.00–8.00	0/1
16	I	Campolieto	S	15	1	0	nd	nd	D	17/05/2011	18.00–8.02	1.5/0
17	P	San Giovanni in Galdo	S	100	1	1	WCB8(3)	wolf	W	21/05/2011	18.00	0/0
18	Q	Castropignano	S	13	2	0	nd	nd	W	25/05/2011	19.00	0/0
19	I	Campolieto	S	15	1	1	DCB6	dog	scavenged	26/05/2011	18.00–8.00	1.5/1
20	R	Busso	S	20	4	4	WCB10(3); WCB9	wolf	W	13/07/2011	21.00–7.00	1/1
21	G	Pietracatella	S	50	4	2	DCB7; WCB11	dog; wolf	W	18/07/2011	21.00–6.00	1.5/1
22	T	Baranello	C	50	4	3	DCB8; DCB9	dog	D	05/08/2011	21.00–6.00	0/1
23	Z	Macchia Valfortore	G	30	1	1	WCB12(2)	wolf	W	08/09/2011	21.00–6.00	1.5/1
24	PB	Pietracatella	S	12	2	2	WCB13(2); WCB12(2)	wolf	W	15/09/2011	19.00	0/0
25	ZL	Mirabello	G	20	6	6	DCB11	dog	W	17/09/2011	3.00	0/2
26	MG	Toro	S	11	1	1	WCB14(1)	wolf	W	04/10/2011	22.00–6.01	nd/2
27	ND	Toro	G	5	1	1	WCB14(1)	wolf	W	04/09/2012	22.00–6.00	nd/2
28	DB	Gildone	S	20	5	0	nd	nd	W	02/05/2013	22.00–6.01	nd/0

depredation patterns between individuals belonging to these related groups, with members of group A actively preying near the villages Gambatesa, Tufara and Riccia while members of group B focused on the area of the villages Pietracatella, Monacilioni, Sant’Elia a Pianisi and Macchia Valfortore (Fig. 1).

The third hypothetical pack (Pack 3, Fig. 3) was localized in the western part of the study area in a territory of about 51 km² and was com-

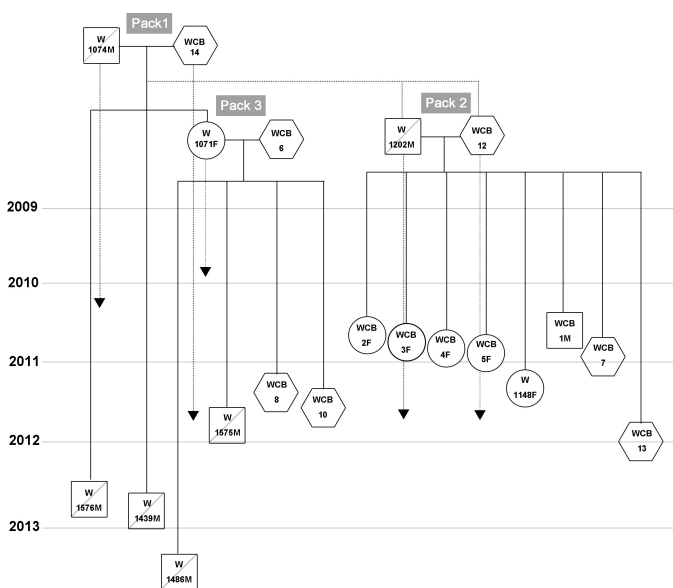


Figure 3 – Genealogies of the three hypothetical wolf packs living in the study area. Males are represented by squares, females by circles and individuals for which it was not possible to detect their gender by hexagons. Thick continuous lines connect reproductive pair members; thin continuous lines include offspring of a same year. Vertical arrows indicate sampling period of each genotype. Slashes indicate found-dead wolves.

posed by WCB6, an individual of unknown familiar origin, and female W1071F, an offspring of pack 1, which showed a likelihood score of 0.966 to be the putative parents of W1486M, W1575M, WCB10 and WCB8.

The remaining 13 genotypes, 11 of which were detected from found-dead individuals and two identified from salivary samples collected on predations, appeared to be unrelated and could not be assigned to any group, thus were considered as potential floaters or unrelated helpers (von Holdt et al., 2008; Caniglia et al., 2014). This category included all the admixed individuals identified in the study area.

Discussion

During its ongoing expansion, the Italian wolf gradually recolonized most of its historical mountain territories and hill zones, reaching areas close to villages and even to large towns (Chapron et al., 2014), but raising social conflicts with local communities (Caniglia et al., 2010). Monitoring projects, targeted to assess wolf presence and distribution, while evaluating predation patterns on domestic animals and their real impact on the livestock raising economy, are becoming a conservational priority, especially in rural zones (Milanesi et al., 2015). Anyway, reliable data about wolf distribution and damages on livestock are seldom available (Magrini, 2014; Marino et al., 2016; Zingaro and Boitani, 2017; Ciucci et al., 2018).

In this work, for the first time, we used salivary swabs collected from preyed livestock together with other opportunistically-collected biological samples such as faeces and carcasses in order to monitor the wolf presence and activity in a small and poorly investigated study area. The genotyping at maternally (mtDNA) and paternally (Y-linked STR) inherited markers and autosomal loci (STRs) of canid DNA allowed to confirm the presence of the wolf in a rural human-dominated landscape of Central Italy (Valle del Fortore, Campobasso Province), characterized by numerous cases of livestock predations but also by high wolf mortality. Our molecular identifications detected 28 wolf genotypes, five wolf-dog admixed individuals and nine dogs in this area. Salivary

swabs provided a substantial contribution to the characterization of the investigated population. Despite their supposed low-quality DNA content, these samples showed genotyping rates close to 60%, considerably higher than those usually obtained from non-invasive sample analyses (Ruiz-González et al., 2013), thus allowing the detection of 51% of the genotypes observed in the study area. Overall, values of genetic variability detected in the monitored wolf population were relatively high and not significantly lower than those observed in the reference population, consistent with the hypothesis of a recent wolf colonization of the study area. Anyway, all these comparisons should be treated with caution because part of the genetic variability in the detected wolves could have been unsampled and such estimates might have been inflated by the limited sample size and by the high number of related individuals sampled within packs.

Admixture identifications

Though none of the found-dead individuals showed morphological anomalies, the assignment procedures detected traces of admixture in five of them. However, they all represented probable cases of backcrossing rather than recent F1 or F2 individuals, because four of them shared from 2.4 to 19.5% dog-derived nuclear genome proportions and one showed only the introgression of a dog haplotype on the Y chromosome, suggesting a more ancient admixture event likely occurred during the initial population re-expansion (Galaverni et al., 2017). However, the admixture cases we identified could have been limited by the restricted number of microsatellites used for the individual genotyping, which allow the reliable detection of hybridization only up to two-three generations in the past (Vähä and Primmer, 2006; Caniglia et al., 2014; Randi et al., 2014). All the detected admixed individuals appeared to be unrelated and none of them was assigned to any of the identified packs, suggesting that backcrossing and the diffusion of dog variants in wolves can be constrained by natural selection and the stability of familial groups, though such hypotheses should be taken with caution given the limited sample sizes and the uncertainty about the actual number of individuals in the study area (Randi et al., 2014; Galaverni et al., 2017). Interestingly, none of them were found to be involved in livestock attacks, weakening the presumption of a higher attitude of wolf-dog hybrids to more likely attack domestic animals, as recently confirmed by Bassi et al. (2017).

Pack reconstructions

The minimum number of individuals we identified in the study area varied from three in 2009 to 13 in 2011, though an unknown proportion of individuals might have gone unsampled because of insufficient sampling and limited amplification success of non-invasive samples (Caniglia et al., 2014; GranrothWilding et al., 2017). However, their spatial and temporal localizations, together with parentage analyses, allowed us to reconstruct the rough genealogies of three hypothetical packs, plus a number of potential floaters. The average size of the home ranges of the identified packs (27–156 km²) was similar to the estimated wolf territories reported in central Italy (50–200 km², Apollonio et al., 2004; Scandura et al., 2011; 38–244 km², Caniglia et al., 2014) which, mainly due to an overall higher prey density and habitat fragmentation, are smaller than in other European (80–300 km²) and North American (100–800 km²) wolf populations (Kusak et al., 2005; Jędrzejewski et al., 2007; Mech, 1999; Fuller et al., 2003). The average pack size (4.0±0.9) was comparable to other estimates for the Italian and other European wolf populations (Fuller et al., 2003; Mech and Boitani, 2003; Nowak et al., 2008; Marucco et al., 2009; Caniglia et al., 2014; GranrothWilding et al., 2017), whereas our proportion of floaters (33%) was slightly higher than those reported in other studies (15%, Fuller et al., 2003; 17%, Caniglia et al., 2014). However, both such estimates are based on a limited number of loci and familial units and could be biased because some packs and genealogies may have been missed. However, although in this study no other field method was practicable at a large scale and we did not obtain independent estimates of the proportion of sampled parents, which are essential for pedigree reliability, the multigenerational pedigrees that we reconstructed indic-

ated that pack composition was variable and dynamic through time in the study area (Fig. 3), which appeared to be initially characterized by the presence of a single pack (Pack 1) that successively produced the other two (Pack 2 and Pack 3). Interestingly, the foundation of Pack 3 involved a female offspring of the original Pack 1 who mated with an unrelated male, coherently with the tendency of the species to avoid inbreeding through male-biased dispersal and pack turnover or splitting (Geffen et al., 2011). However, Pack 2, located in the core of the study area, was established by a brother-sister pair, indicating that particular conditions, such as high mortality rates and scarce availability of unrelated mates due to the recent recolonization, can favor incestuous matings in wolves (von Holdt et al., 2008; Rutledge et al., 2010). Anyway, we are conscious that our pack reconstructions are not conclusive because we could have not sampled all the members of the familial groups, although we are confident to have identified all the possible packs living in the study area, as confirmed by the other monitoring tools that were occasionally employed (D'Amico, 2014). Our genealogies, even if preliminary, allowed us to evaluate local scenarios of turnover, inbreeding and dispersal rates that could be used as a baseline for the evaluation of the local hunting patterns on livestock and to identify adequate managements actions.

Possible livestock predation patterns

In this case-study, molecular analyses allowed the identification of the attacker profile in 68% of the killed animals and the reconstruction of the possible predation patterns in 62% of the monitored attacks. Preliminary veterinary field reports were always confirmed by molecular identifications, with the exception of six samples from farm ZL, which showed wolf-like predation patterns but provided only DNA traces of dog, probably due to post-mortem consumptions by individuals that acted as scavengers. However, dogs were directly responsible for at least 14% of the attacks, corresponding to the loss of 23% of livestock heads. These data, though limited in time and space, confirmed that free-ranging dogs might represent a relevant threat for farming activities, contributing to exacerbate hostile attitudes towards the wolf (Home et al., 2017; Wierzbowska et al., 2016).

Molecular identifications detected that most of the livestock predations (52%) were perpetrated by wolves, whose attacks were focused on sheep and generally occurred in the spring-summer period, always during the night, confirming previous findings on wolf predatory behaviour towards domestic prey conducted in the Northern Apennines (Mech and Boitani, 2003; Russo et al., 2014; Dondina et al., 2015).

Integrating depredation patterns with pack reconstructions we found that most of the attacks (87%) were carried out by individuals belonging to a pack and only one by a wolf unrelated to any identified pack. Observing pack-specific patterns, we reconstructed three possible scenarios: I) In Pack 1, the hypothetical reproductive female (WCB14) committed two attacks in different farms after the death of the putative reproductive male (W1074M) and two of their offspring (W1439M and W1576M, found dead several kilometers far from depredation sites), suggesting that the pack destructuring might have reduced the predation efficiency of the remaining wolf, which subsequently focused on livestock predations in an area with high farm density (Wielgus and Peebles, 2014; Imbert et al., 2016). II) In Pack 2, conversely, 80% of the members were involved in depredations, indicating a recurrent livestock hunting strategy. Interestingly, the hypothetical parental female of this pack (WCB12) was the most active individual responsible for three attacks, in one of which she cooperated with an offspring, suggesting a cultural transmission of the predatory behavior. Such tendency might have been favored by the absence of effective precautionary measures in the attacked farms, such as trained dogs, electrified fencing or night stables (Dalmaso et al., 2012; Dondina et al., 2015; Ciucci et al., 2018). If true, such hypothesis might have important livestock management repercussions because farmers might avoid such a learning predatory strategy by the adoption of adequate and active defense (Dalmaso et al., 2012; Dondina et al., 2015; Ciucci et al., 2018). III) In Pack 3, two related members were responsible for a single at-

tack: one acted alone while the other cooperated with an individual of unknown origin, maybe a floater or an adoptee.

Management Implications

Wolf depredations resulted in the loss of about 4%, on average, of the livestock patrimony of the monitored farms, facilitated by the limited presence of adequate prevention measures, though in some cases the attacks were confused with those performed by other carnivores (Harms et al., 2015). Nonetheless, the regional legislation compensates for livestock loss or damages caused not only by wolves, but also by stray dogs, bears and eagles. In this way, despite its limited extension, in the period 2000–2013 the Campobasso Province indemnified 622 declared wolf depredations (5838 heads) spending 476856.33 €, with an average cost per year of 20000 €. Anyway, in the same period none of the breeders required nor adopted adequate prevention measures (such as electric fences, night shelter, trained dogs, acoustic devices) and livestock predations continued, demonstrating that compensation measures alone cannot be a long-term sustainable strategy because they do neither limit damages nor mitigate conflicts (Boitani et al., 2010; Dondina et al., 2015; Marino et al., 2016; Zingaro and Boitani, 2017). For these reasons, based on the results of this work, we encourage the promotion of effective local, Regional and National damage prevention plans that provide long-term incentives for the adoption of prevention tools and adequate husbandry practices that could significantly reduce damages and compensation costs (Dalmaso et al., 2012; Reinhardt et al., 2012).

Conclusions

Reliable reconstructions of the minimum number of wolf individuals, pack localizations and dynamics, though challenging to obtain, are key parameters to understand the biology and ecology of large carnivores, evaluate their effects on human activities and ensure their long-term conservation (Chapron et al., 2014). In this work we demonstrated that, thanks to the availability of genetic databases of wolf and dog reference genotypes, forensic genetic data obtained analysing carnivore DNA found on predated livestock, integrated with those obtained from the analysis of other kinds of biological samples collected systematically (faeces) or opportunistically (found dead animals), can provide a useful amount of information about presence, distribution and pack structure of the wolf population living in a certain area, and contribute to clarify their impact on herd losses and on the husbandry economy. Such information can be collected with relatively low sampling efforts and costs, largely overlapping with those employed for the analyses of attacked livestock, providing basic data that could be used to design wolf management plans in a human-dominated territory with a heavy wolf mortality and high livestock predation risk. However, such information should be always integrated with additional data obtained from more extensive and long-term wolf monitoring projects, planned in both the core area of the wolf distribution range and in marginal, recently-colonised areas, to better investigate wolf expansion and distribution, monitor the presence of wolf-dog hybridization and illegal killing rates, that still represent major threats to the survival of the species, while preventing excessive damages to human activities where farming practices have abandoned traditional prevention measures. ☞

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Genetic variability at 12 autosomal short tandem repeat (STR) loci in reference wolves, wolves sampled in the study area, reference dogs, and dogs sampled in the study.