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Case report

Who is who? Identification of livestock predators using forensic genetic approaches

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ABSTRACT

Molecular identifications of salivary DNA are increasingly applied in wildlife forensic investigations, and are successfully used to identify unknown livestock predators, or elucidate cases of large carnivore attacks to humans. In Europe most of livestock predations are attributed to wolves (*Canis lupus*), thought free-ranging dogs are sometime the responsible, and false predations are declared by breeders to obtain compensations. In this study we analyzed 33 salivary DNA samples collected from the carcasses of 13 sheep and a horse presumably predated by wolves in seven farms in central Italy. Reliable individual genotypes were determined in 18 samples (corresponding to samples from nine sheep and the horse) using 12 unlinked autosomal microsatellites, mtDNA control-region sequences, a male-specific ZFX/ZFY restriction-site and four Y-linked microsatellites. Results indicate that eight animals were killed by five wolves (a male and four different females), the ninth by a female dog while the horse was *post-mortem* consumed by a male dog. The genotype of one female wolf matched with the genetic profile of a female wolf that was non-invasively sampled 4 years before in the same area near livestock predation remains. Genetic identifications always supported the results of veterinary reports. These findings show that salivary DNA genotyping, together with detailed veterinary field and necropsy reports, provides evidence which helps to correctly identify species, gender and individual genetic profiles of livestock predators, thus contributing to clarify attack dynamics and to evaluate the impact of wolf predations on husbandry.

1. Introduction

The conservation of large carnivores (brown bear *Ursus arctos*, wolf *Canis lupus*, lynx *Lynx lynx*, and wolverine *Gulo gulo*) in developed European countries is strictly conditioned not only by habitat and prey availability, but also by conflicts with local communities and stakeholders: livestock breeders and hunters [1,2]. Conflicts are mainly caused by predations on domestic ungulates, or by presumed competition with hunters for the same wild ungulate prey [3]. Conservation plans pay particular attention to minimize damages through prevention measures or economical compensations [4]. Unfortunately, these policies are not always efficient, and they are sometime implemented without the necessary technical expertise [5]. Thus, stakeholder pressures fuel uncontrolled illegal killings of wild carnivores, which are almost everywhere protected by national legislation [6,7]. Negative public opinion [8] might, moreover, push managers and politicians to launch more drastic predator control plans [9,10].

The wolf is the most widespread large carnivore in Europe [11]. After centuries of population decline and range contraction, wolves are now expanding recolonizing parts of their historical ranges, reappearing not only in natural areas, but also in developed agricultural regions [12–14]. Wolves preferentially predate on wild animals [11,15], but, when available, they certainly do not ignore domestic ungulates [16]. It is estimated that about 10,000 livestock per year are killed by wolves in Europe [17]. Wolf predations might be confused with those of other carnivores, mainly free-ranging domestic dogs, which are widespread in south European countries [18]. Dog predations might be ascertained through skilful evaluations of the evidence collected on killing sites, through the identification of scat or hair samples, the size and spacing of bite wounds on prey, and the behavioural pattern of the attacks [19]. However, confusing field conditions or the insufficient skill of technicians makes it often difficult to obtain correct identifications [20]. In these cases, wolves are innocently blamed, exacerbating the anger of stakeholders and managers. Moreover, some countries generically compensate for canid damages,

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regardless of whether predators are wolves or dogs [17]. These strategies are very inefficient, because compensation costs significantly increase and breeders are encouraged to report false predations. Sound management plans, targeted to minimize frauds and reduce compensation costs, require realistic evaluations of the impact of wolf predations. Thus, there is a need to implement adequate procedures for the identification of predators. Recent developments in forensic veterinary medicine and non-invasive genetics can offer a contribution [21–25].

Molecular techniques and genetic databases are being used to identify species, individuals and gender in DNA extracted from biological samples such as hair, faeces, urine and blood traces, collected in and near predation sites [26], and from saliva samples collected from bitten skins [27–29]. Here we report the results of a case-study planned to evaluate the power of non-invasive molecular genetic methods to identify the predator species, if wolves or dogs, in areas of the Italian Apennines. We collected salivary DNA from the bodies of 13 killed sheep and a horse. They were genotyped at the mtDNA control-region (diagnostic for the Italian wolf population [30]), 12 unlinked autosomal microsatellites (which are informative to identify wolves, dogs and their first two generation hybrids [31]), a male-specific restriction-site of the ZFX/Y gene (used to sex samples [32]), and four Y-linked microsatellites (used to identify paternal autosomal haplotypes [33]). Multilocus genotypes were matched to a large database of Italian wolf and dog genotypes (the ISPRA *Canis* database; [34]) and used to: (i) identify their population of origin: dogs, wolves or hybrids; (ii) estimate the minimum number of individuals presumably involved in the predation events; and (iii) search for matches between salivary DNA and the genetic profiles of the livestock-guard dogs owned by attacked farmers.

2. Materials and methods

2.1. Sample collection

Salivary swab samples were collected from bite wounds on 10 sheep and three lambs that were killed or injured by unidentified canids during seven attacks that occurred from February to August 2010 in six different farms (named A, B, C, D, E and F in Table 1) in central Italy (Molise Region, Campobasso Province). In another farm (G) we collected salivary swab samples from the body of a female horse that, according to the breeder, was killed by wolves. However, the veterinary necropsy showed that the horse died due to natural causes (intestinal obstruction) and it was apparently *post-mortem* consumed by unknown carnivores. The sheep farms had between 3 and 150 heads of livestock and, with the exception of farm G, were protected by fencing shelters 1.5–2.0 m high. Farms B, D, G used also trained guard dogs (Table 1).

Predators' bites were identified by the presence of sub-dermal haemorrhages behind the wounds. It is well known that, in contrast, consumers' bites do not show such kind of haemorrhages [35]. We collected from one to six samples per wound for a total of 33 samples (Table 1), using dry sterile cotton swabs, rubbed around the edge of the bites, that were stored dry. Skin and tissue fragments were cut around the bites too, and stored in 10 volumes of 95% ethanol. Samples from multiple bites in the same carcass were collected and stored separately. All samples (except in farm E) were collected from killings that occurred less than 36 h before the inspection. Salivary DNA degradation was assessed by replicating sampling from two injured sheep in farm F after ca. 72 h. Finally, we collected three mouth swabs from the guard dogs in farms B and D to check for matches with the putative predators. Unfortunately we were not permitted to sample the two guard dogs in farm G, where the horse died.

Detailed veterinary reports were compiled, reporting descriptions of predation patterns, location, size and shape of bite wounds in prey bodies, results of necropsies, presumed day of death, identification of putative predators, exclusion of alternative causes of death, evidences of *post-mortem* consumption by scavengers [19,35]. All samples were collected, shipped to the laboratory and genetically analyzed blind, without any reference to the results of the veterinary reports. Molecular and veterinary identifications were compared only at completion of the laboratory analyses.

2.2. Microsatellites and mtDNA genotyping

Total DNA was extracted using the Zymo Research Quick-gDNA™ MiniPrep kit (Zymo Research Corporation, USA), with the following two small variations of the standard protocol described by the manufacturer: (1) to improve DNA quantity and quality, the extractions were preceded by a pre-digestion step; each sample was digested at 56 °C for 45 min in a lysis buffer containing 175 μL white cell lysis buffer (WCLB), 20 μL proteinase *K* (for the enzymatic digestion of membranes and other protein structures) and 5 μL 10% sodium dodecyl sulphate solution (SDS; an anionic detergent that solubilizes cell membranes and denatures the proteins) and (2) the DNA samples were recovered through a final elution in 100 μL of 10 mM TE (Tris–EDTA) buffer.

Each DNA sample was used to PCR-amplify 12 canine unlinked autosomal microsatellite loci (STR; short tandem repeat): seven dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; C09.250 and C20.253) and five tetranucleotides (FH2004, FH2079, FH2088, FH2096 and FH2137), selected for their high polymorphism and reliable scorability in wolves and dogs [36], and used for individual identification in a long-term non-invasive monitoring project of the Italian wolf population and in forensic applications [23,34,36]. Preliminary testing showed that these markers do not amplify domestic or wild ungulate DNA [32]. The probability-of-identity [37], that is, the probability of finding, by chance, more than one individual with the same genotype (shadow effect; [38]) with this panel of 12 STR loci was: PID = 3.2×10^{-10} (among unrelated individuals), PIDsibs = 1.1×10^{-4} (among full sibs) as estimated using 1086 not related Italian wolf genotypes (these probabilities were not corrected for substructure, which was never detected in the recently expanding wolf population of the Apennines [11]); and PID = 5.3×10^{-12} and PIDsibs = 4.2×10^{-5} in 405 dogs. Samples were sexed by a PCR-RFLP assay of the zinc-finger protein genes ZFX/Y [32], and paternal haplotypes, characterized by different allele frequencies in dogs and wolves [39], were identified by four Y-linked STR (MS34A, MS34B, MSY41A and MS41B [33]).

DNA samples were genotyped using a multiple-tube protocol requiring from four to eight independent amplifications per locus [36,40]. After the first four replicates, samples showing ≤50% positive PCR (PCR+) over a total of 48 amplifications at the 12 autosomal STR loci were discarded. A reliability analysis (with software Reliotype; [41]) was performed on samples showing >50% PCR+, and unreliable loci (at threshold $R < 0.95$) were additionally replicated four times. Only complete genotypes, which obtained a final $R > 0.95$ were definitively accepted. Consensus genotypes were reconstructed using Gimlet v. 1.3.3 [42] and the following rules: heterozygotes were accepted only if the two alleles were seen at least in two replicates; homozygotes were accepted only if one and only one allele was seen at least in four replicates. The consensus genotypes were matched among them and with the ISPRA *Canis* database, using GenAlEx v. 6.4 [43].

The panel of autosomal and Y-linked STR loci was amplified in seven multiplexed primer mixes using the QIAGEN Multiplex PCR Kit (Qiagen Inc., Hilden, Germany), an Applied Biosystems Thermal Cycler (ABI GeneAmp® PCR System 9700) and the following thermal profile: 94 °C/15 min, 94 °C/30 s, 57 °C/1 min and 30 s,