Forensic Science International: Genetics 4 (2010) 334–338

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Case report

Forensic DNA against wildlife poaching: Identification of a serial wolf killing in Italy

Romolo Caniglia *, Elena Fabbri, Claudia Greco, Marco Galaverni, Ettore Randi

Institute for Environmental Protection and Research (ISPRA), Laboratory of Genetics, Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy

ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form 14 October 2009
Accepted 27 October 2009

Keywords:
Canis lupus
Wildlife poaching
Microsatellite
mtDNA control-region
Bayesian analysis
Non-invasive sample

ABSTRACT

The recent expansion of the Italian wolf population through the Apennine and western Alps, after centuries of contractions, is causing conflicts with human activities leading to a rise in poaching or illegal killings. Here we show how molecular population genetics has been used to identify a suspect serial wolf killer. We analysed DNA extracted from a necklace made of ten presumed wolf canine teeth, confiscated in 2008 to a man living in the northern Italian Apennine (Liguria Region). Individual genotypes were determined using 12 unlinked autosomal microsatellites (STRs), mtDNA control-region sequences, a male-specific ZFX/ZFY restriction-site and three Y-linked STRs. Results indicate that the teeth belonged to six different individuals (three males and three females), which were assigned to the Italian wolf population with $p > 0.90$ by Bayesian procedures. One of these genotypes matched with the genetic profile of a male wolf previously found-dead and already non-invasively sampled in the same area. Another genotype matched with that of a female wolf non-invasively sampled twice in the same area 1 year before. These data are being used as forensic genetic evidence in the ongoing criminal trial against the suspect serial wolf killer.

1. Introduction

After centuries of population decline and worldwide range contraction due to habitat changes, decline of natural prey species and direct persecution by humans, the wolf (*Canis lupus*) is expanding again in Italy and other parts of Europe [1]. Wolves in Italy were confined south of the Po River since the turn of the last century, and less than 100 individuals survived in the 1970s in two fragmented areas in the central-southern Apennine [2]. This declining demographic trend quickly reversed in the 1980s, when wolves started to expand in parts of their historical range in the Apennine, reaching the south-western Alps, France and Switzerland [3–5]. The return of wolves in anthropic areas is fuelling conflicts with local communities, mainly with hunters and livestock breeders. While hunters wrongly maintain the idea that wolves are competitors for the same wild ungulate game (wild boar, red deer, roe deer and fallow deer), livestock breeders some time really suffer significant economical losses caused by predations on domestic herds. Although both national and local authorities have activated damage prevention and compensation policies, their actions are rarely enforced rapidly and efficiently. Consequently, and despite legal protection accorded to the wolf since 1976, poaching and various forms of illegal killings are widely practised [6]. An estimated 20% of the total wolf population (numbering *c.* 800 animals; [1]) is illegally or accidentally killed every year in Italy [6]. In addition to intentional shooting and poisoning, wolves are accidentally, but always illegally, killed by poisoned baits against foxes and small carnivores, or by snares for wild boars. Hence, poaching is widespread and perhaps remains the major threat to wolf survival [7,8]. Nevertheless, poachers in Italy have never been identified and prosecuted by law, so far.

Here we describe how molecular genetic identification methods are being used to contrast the illegal killing of wolves in Italy. In 2008 the Provincial Police of Genova confiscated a necklace (Fig. 1) made by ten canine teeth to a man living in a small village in the northern Italian Apennine, Liguria Region, Genova Province. After a few days the Provincial Police discovered in the same area a male wolf carcass without the entire muzzle. The necklace and wolf tissue samples were sent to the Laboratory of Genetics of ISPRA (Institute for Environmental Protection and Research) where DNA was extracted and multilocus individual genotypes were determined at the mtDNA control-region [9], 12 unlinked autosomal microsatellites [10] and three Y-linked STRs [11], and sexed using a male-specific ZFX/ZFY restriction-site [12]. The genotypes were matched with a large database of wolf and dog genotypes that is being implemented at ISPRA, in compliance with European Community and national laws that require that wolf populations, as well as other protected large predators (brown

* Corresponding author. Tel.: +39 0516512251; fax: +39 051796628.
E-mail address: romolo.caniglia@gmail.com (R. Caniglia).

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doi:10.1016/j.fsigen.2009.10.012

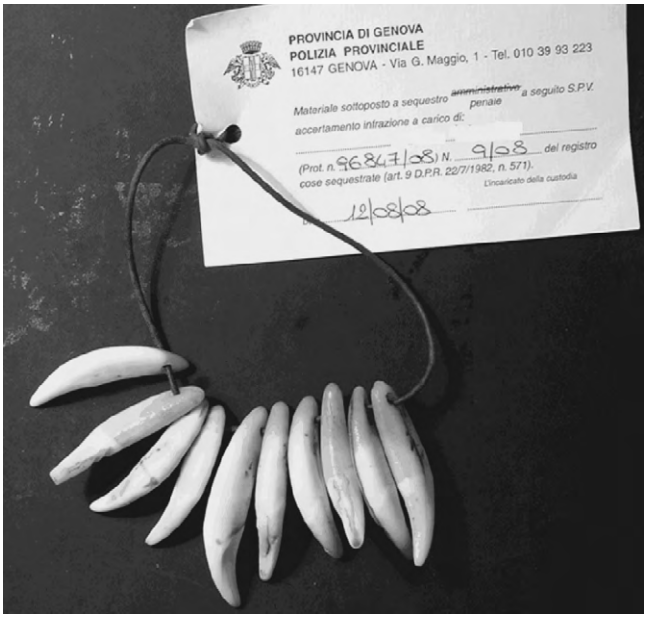

Fig. 1. The confiscated wolf tooth necklace.

bear, lynx), are monitored [6,13]. The Italian wolf database consists of multilocus genotypes obtained from DNA extracted from found-dead wolves collected throughout the entire Italian wolf range distribution in the last 15 years ($n = 417$), from non-invasive samples (scats) collected during a monitoring project in the Apennine from 2000 to 2009 ($n = 341$ [14]) and the western Alps from 1999 to 2004 ($n = 130$) [4]).

The database is used by managers to obtain detailed information on wolf presence, distribution range, population size and structure in the northern Apennine and western Alps [14]. These data were analysed aiming to: (1) identify the species and the population of origin of each sample, either if wolf or dog; (2) determine the individual genotypes and count the number of individuals to which the ten teeth belonged; (3) assess any eventual match between each tooth genotype and the found-dead wolf; and (4) search the database for additional matches with wolves that were non-invasively identified during the ongoing population monitoring project.

2. Materials and methods

2.1. DNA extraction and molecular analyses

DNA was extracted from a small fragment of muscular tissue (stored at −20 °C in 10 volumes of 95% ethanol) of the wolf carcass, and from dental pulp samples obtained by slow drilling the roots of the confiscated teeth, using a guanidinium-silica protocol [15]. All DNA samples were PCR-amplified using canine specific primers for: (1) 350 bp of the mtDNA control-region, which contains diagnostic mutations for the identification of the Italian wolf haplotype W14 [9]; (2) 12 unlinked autosomal microsatellites, including seven dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; [16]; C09.250 and C20.253; [17]), and five tetranucleotides (FH2004, FH2079, FH2088, FH2096 and FH2137; [18]), that were selected for their high polymorphism in the Italian wolf population [10]. This panel of microsatellites allows determining the individual genotypes with probability of identity PID = 7.1×10^{-9}, and expected PID among full sib dyads PIDsibs = 3.1×10^{-4}, in the Italian wolf population [19,20,4]. The genotypes were sexed by PCR-RFLP of diagnostic ZFX/ZFY sequences [12,20] and male individuals were also amplified at three Y-linked microsatellites: MS34A, MS34B, MS41B [11]. PCR-amplifications were carried out in 10 μl reactions, using respectively 1 μl or 2 μl DNA solutions from tissue or tooth extractions, plus 2 μg of BSA, and were optimised for each primer pair and for tissue or tooth samples (protocols are available upon request). Tooth pulp and tissue samples were extracted and amplified in dedicated separate rooms under sterile UV laminar flood hood, and pulp samples genotyped using a wolf-specific non-invasive multiple-tube protocol [14]. Negative (no DNA in PCR) and positive (samples with known genotypes) controls were always used. PCR products were analysed in an automated sequencer ABI 3130XL (Foster City, CA), using the software Sequencing Analysis v.3.7 and Seqscape v.2.5 for sequences, and Genescan v.3.7 and Genmapper v.4.0 for microsatellites.

The quality of tooth DNA was initially screened by four replicated PCRs of two microsatellites (FH2096 and FH2137). Only those samples showing more than 50% positive PCRs (*i.e.*, PCRs producing the expected amplicons) were further amplified four times at each of the remaining ten microsatellites and sexed. The software Reliotype was used to assess genotype reliability [21], and unreliable loci (at score threshold $R = 0.95$) were additionally replicated other four times. All those samples that were not reliably typed at all loci after eight PCR replicates were definitively discarded. Consensus genotypes were reconstructed using the software Gimlet v.1.3.3 (http://pbil.univ-lyon1.fr/software/Gimlet/gimlet.htm) [22], accepting heterozygotes only if the two alleles were seen at least in two replicates and homozygotes only if the allele was seen at least in four replicates. Individual genotypes were recorded in Excel and the software GenAlEx v. 6.1 (http://www.anu.edu.au/BoZo/Genalex) [23] was used to estimate the values of population genetic parameters.

2.2. Bayesian admixture analyses

The software Structure v. 2.2 [24] was used to assign individuals to baseline wolf or dog populations, independent of any prior non-genetic information. The baseline wolf population included the genotypes determined in 176 randomly selected tissue samples obtained from found-dead wild-living wolves that were accidentally or illegally killed in Italy. All these animals had the typical Italian wolf coat colour pattern and did not show any detectable phenotypic and genetic signals of hybridisation [9,10,25]. The baseline dog population was composed by the genotypes determined in 118 blood samples collected from dogs living in rural areas in Italy. We run Structure with five repetitions of 10^5 iterations following a burn-in period of 10^4 iterations, selecting the "admixture model" (each individual may have ancestry in more than one parental population) and the "*I model*" (independent allele frequencies). According to previous studies [10,25] the optimal number of populations was set at $K = 2$, the value that maximised the posterior probability of the data. At $K = 2$, we assessed the average proportion of membership (Q_i) of the sampled populations to the inferred clusters. Then, we assigned each individual genotype to one cluster if the proportion of membership was $q_i > 0.90$, or to both clusters if the proportion of membership was $q_i < 0.90$ (admixed individuals). Individual multilocus scores were computed using Genetix v.4.05 (http://www.genetix.univ-montp2.fr/genetix/genetix.htm) [26] and patterns of differentiation were visualized by Factorial Correspondence Analysis FCA [27].

3. Results and discussion

3.1. Genetic identifications

We obtained clean mtDNA sequences from nine of the tooth samples and from the muscle. These sequences were aligned