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Case report

Wildlife molecular forensics: Identification of the Sardinian mouflon using STR profiling and the Bayesian assignment test

Rita Lorenzini [a,*], Pierangela Cabras [b], Rita Fanelli [a], Giuseppe L. Carboni [c]

[a] Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Via Tancia 21, 02100 Rieti, Italy
[b] Istituto Zooprofilattico Sperimentale della Sardegna, Via Aresu 2, 08048 Tortolì, Ogliastra, Italy
[c] Servizio Territoriale Ispettorato Ripartimentale del Corpo Forestale e di V.A. di Lanusei (C.F.V.A. di Lanusei), Via Ilbono 9, 08045 Lanusei, Ogliastra, Italy

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ABSTRACT

A forensic short tandem repeat (STR) typing test using a population database was developed to investigate an instance of poaching on the protected Sardinian mouflon. The case study involves a suspected poacher found in possession of a carcass, which he claimed was that of a sheep from his flock and had died accidentally. His claim was refuted by the molecular forensic analyses as DNA typing and the Bayesian assignment test revealed the carcass to be mouflon-derived; the genetic profile of the carcass matched also that of additional trace evidence collected by forestry officers at the scene of the kill. The matching evidence led to the poacher being charged with the illegal harvest of protected wildlife. Molecular techniques, in combination with a reference population database, and the appropriate statistical evaluation of genetic information, are fundamental to wildlife forensics. This approach allows DNA testing to be accepted in court as submissible evidence in the fight against poaching and other crimes involving wildlife.

1. Introduction

Within the Mediterranean region populations of the mouflon (*Ovis aries musimon*) are restricted to the islands of Cyprus, Corsica and Sardinia, and to small areas of Turkey and the Middle East [1]. The additional populations of the mouflon found today across much of continental Europe stem from introductions commenced in the mid 1700s [2,3]. By the 1960s, however, the mouflon of Sardinia faced extinction; unrestrained hunting was the cause lowering the population to around 400 [4]. In the decades since, and following the implementation of protective legislation, both at the national and local level, mouflon numbers have revived and now stand at approximately 3000 [3]. Although the Sardinian mouflon is protected under the Bern Convention and by the Italian law, as an Especially Protected Species (National Law 157-1992), it is listed as "vulnerable" on the IUCN (International Union for Conservation of Nature) Red List of Threatened Animals [5]. In spite of this legal protection and its recent increase in numbers, the mouflon in Sardinia remains under constant threat: from roaming dogs, crossbreeding with domestic sheep (*Ovis aries aries*), and continued poaching (still the most significant cause of mouflon mortality).

Wildlife poaching is a scourge worldwide. Species either endangered or close to extinction cannot escape illegal harvesting even when they are maintained in 'safe refuges', including protected areas and sanctuaries [6,7]. In the fight against poaching all known investigative tools need to be deployed to enable law enforcement agencies to prosecute the perpetrators. Nowadays, molecular forensic methods are being used increasingly to underpin those anti-poaching laws that deal with the illegal procurement of, or trade in, protected wildlife [6,8–10]. Accurate species identification based on the mitochondrial or nuclear DNA analysis from animal tissue, teeth, ivory, scales [11–13] and other biological trace elements [7] is now conducted routinely in forensic laboratories worldwide. In addition, genetic methods are used for determining sex [14,15], assessing paternity and kinship [16], establishing the source population of single individuals [7,13], and recognizing hybrids [17] and crossbreds [18]. Forensic DNA typing, using highly polymorphic short tandem repeat (STR) molecular markers, is of particular value for matching individual specimens to other bits of evidence that may be found in association with an illegal kill, but which are unidentifiable in the phenotype.

In wildlife and human forensics the strength of the DNA evidence needs to be assessed using a rigorous statistical approach before it can be deemed acceptable for submission to a court of law. Reference population databases are essential to this task as they

* Corresponding author. Tel.: +39 0746201599; fax: +39 0746201642.
E-mail addresses: rita.lorenzini@izslt.it (R. Lorenzini), pierangela.cabras@izs-sardegna.it (P. Cabras), rita.fanelli@izslt.it (R. Fanelli), glcarboni@regione.sardegna.it (G.L. Carboni).

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provide the allele frequencies (and other genetic parameters) needed for determining probability of identity, random match probability, paternity, and kinship indices [19,20]. Despite issues inherent to wildlife, human forensic guidelines [21,22] should be followed. While population databases are requisite to genetic forensic analyses, many of the species targeted by wildlife criminals are either highly protected, rare, or elusive, which renders the acquisition of representative genetic information for these databases difficult. Indeed, the lack of genetic population data is one of the major challenges facing the wildlife forensic scientist today. This is the first time that an STR system-based database is provided for the Sardinian mouflon, and used for forensic purposes.

Here we report on a case involving the poaching of a mouflon on the island of Sardinia in 2009. Using molecular techniques, and the Bayesian assignment test, our analyses attempted to: (1) identify the source species of a carcass that was suspected to be that of an illegally harvested animal, and (2) prove that the genetic profile of the carcass matched that of trace evidence left by the poacher, in order to be able to link him unequivocally to the scene of the crime.

2. Case history

It is alleged that a sheep breeder illegally hunted a mouflon in the province of Ogliastra (Sardinia). At the kill site he removed the skin, viscera and all other parts of the carcass that could have helped trace it back to the original taxon (Fig. 1), before he hid the carcass in his truck and fled the area. Soon after he was stopped by local forestry officers manning an anti-poaching roadblock. Here, the officers discovered the carcass hidden behind the driver's seat, but found no weapons. Upon being questioned the breeder argued the carcass was that of one of his sheep that had died accidentally. After seizing the carcass the forestry officers detained the suspect and returned with him to the sheepfold to look for signs that would either confirm or rebut his statements. There, they found small stains of fresh blood mixed with soil left on stones, leaves and twigs. Subsequently, the carcass and the bloodstains were submitted to our laboratory for necropsy and for molecular analysis, either to support or refute the breeder's claims. Upon necropsy (Fig. 1) the carcass showed evidence of gunshot wounds; the molecular results revealed it to be that of a mouflon. Furthermore, a positive match was obtained also between the carcass and the various bloodstains collected at the sheepfold. The cumulative evidence led to the suspect being charged with the illegal killing of protected wildlife. The court case remains ongoing.

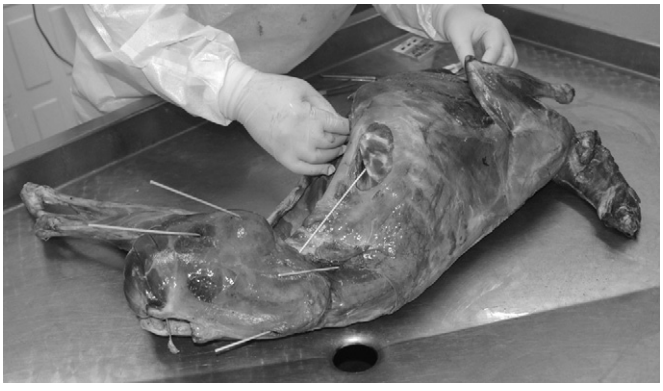

Fig. 1. The carcass of the mouflon seized in 2009 from a suspected poacher, Ogliastra, Sardinia. Gunshot wounds are indicated by the little sticks.

3. Materials and methods

3.1. Laboratory methods

For the allele frequency database whole blood was taken from 96 sheep sourced from a number of farms in Sardinia; over a number of years (2003–2009) 58 samples (either of muscle or blood) were obtained from mouflon that had either died accidentally or had been examined live by veterinarians.

DNA was extracted using the QIAamp Tissue and the QIAamp Blood Mini kits (Qiagen) following manufacturer's instructions. Casework samples consisted of dried bloodstains removed from the surface of leaves, twigs and stones, and of muscle from the carcass. DNA from trace evidence (three stains each from a stone, a leaf, and a twig) was isolated during a separate extraction session, conducted in a dedicated room (low DNA extraction laboratory) using the DNA IQ™ Casework Sample Kit and the Maxwell 16 LEV System (Promega). Two mock extractions (reagents without DNA added) were included during each extraction round to monitor laboratory contamination.

All specimens used for the reference population databases were genotyped at 16 ungulate-derived microsatellite loci, and were chosen for their levels of polymorphism and possibility for multiplexing. For sheep the selected microsatellite loci occur either on different chromosomes, or are located on the same chromosome, but separated sufficiently to render linkage disequilibrium due to non-random association negligible. For detailed information on microsatellite loci see the respective FAO/ISAG and sheep genome map sites http://www.isag.org.uk and http://www.livestockgenomics.csiro.au.

One 5-STR multiplex PCR was optimised to amplify the loci OarCP0049, CRSD0247, INRA0063, HSC and MAF0214; two 4-STR multiplexes amplified OarFCB0020, D5S2, INRA0023 and McM0527, and INRA006, INRA172, ILST0087 and INRA0005, respectively; one 3-STR multiplex amplified McM42, TGLA53 and INRA49. Fluorescent labelling and reaction conditions of multiplexes are available upon request from the authors. 1.5 μl of diluted PCR product was mixed with 15 μl HD formamide, heated at 95 °C for 2 min, kept at 4 °C on a thermal cycler for at least 2 min, and loaded onto an ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems). Fragment sizing was performed using GeneMapper software version 4.0. STR morphologies were scored visually by two persons. The PCR protocol was used to genotype mouflon and sheep samples for the population databases, and the casework samples. Each PCR run included extraction and amplification negative controls. To avoid cross contamination, no positive controls were used during amplification of the casework samples.

3.2. Population genetic analyses

Genetic parameters for the reference populations (allele frequencies, deviations from Hardy–Weinberg Equilibrium, HWE, and tests for linkage equilibrium between pairs of loci) were calculated using Genepop software version 4.0.1 [23] and Genetix software version 4.0.5 [24]. A sequential Bonferroni correction for multiple comparisons [25] was applied in the exact test [26] for deviations from HWE, and in the linkage disequilibrium test. We also estimated the F_{IS} and F_{ST} coefficients [27] to evaluate the levels of inbreeding and differentiation between the sheep and mouflon populations.

In order to assign the carcass to its correct source population we applied the model-based clustering algorithm as used in the software programme Structure version 2.3.2 [28]. This Bayesian method uses multilocus genotypes to identify clusters of genetically similar individuals without assuming a particular mutation process; K theoretical populations that are in HWE, and

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showing linkage equilibrium between loci, are inferred. If populations are genetically differentiated, individuals are assigned in the absence of prior non-genetic information about population affiliation. The probability for each value of K was calculated from the estimated posterior likelihood of the data. If the entire gene pool contains differentiated populations, the probability assumes the highest estimate with the most probable value of K. The average proportional membership (q) was derived for each of the K theoretical populations. A threshold $q_i > 0.90$ was set to include individuals in their own population (see Section 4). Four independent runs of $K = 1–3$ were performed, using 10^5 iterations and a burn-in period of 10^4 iterations. We selected the following options available in Structure: (1) the admixture model (which allows for ancestry of single individuals in more than one inferred population), (2) the mode for correlated allele frequencies (which assumes that for some past generations after population split, the allele frequencies in each population are correlated with those of the ancestral population), (3) no prior population information (which considers all individuals as belonging to one gene pool, without *a priori* defined populations). This last option was recommended by Ball et al. [29] for forensic analyses.

The weight of DNA evidence was tested through calculation of the average probability of identity (P_{ID}) and the random match probability (RMP) [21] using the Gimlet software version 1.3.3. [30] and API-CALC version 1.0 [31]. Estimates of P_{ID} were derived to assess the probabilities of two individuals drawn at random from the population showing identical multilocus genotypes by chance. Concurrently, the number of selected loci was evaluated to check whether it yielded an acceptably low P_{ID} value for our mouflon population, thereby providing sufficient discriminatory power for identifying individuals during the forensic analysis. We used both the standard equation for estimating P_{ID} for co-dominant loci [32] and the equations for P_{ID} that account for parent/offspring and for sibling relationships [33]; this last has been suggested as a conservative upper bound for individual identification in bottlenecked inbred wild populations [34]. In addition, the probability of identity was derived including the F_{IS} inbreeding coefficient obtained for the mouflon population. In human forensics inbreeding is low playing a negligible role in match probability equations; however, in wild species mating system, founder effect and small population size can influence significantly the levels of inbreeding, so that estimates of F_{IS} should be included when calculating the parameters for evaluating the weight of DNA evidence for forensic purposes.

Finally, the RPM was calculated to estimate the probability that a mouflon other than the carcass, and randomly selected from the population, will have the exact genetic profile of the bloodstains found around the suspect's sheepfold; the RPMs were derived for unrelateds, for parent/offspring pairs, for siblings, and, additionally, using the estimated F_{IS} value.

4. Results and discussion

4.1. Species identification

The complete 16-loci profiles obtained from both the sheep and the mouflon reference samples were used to develop their respective population databases. This preliminary step was needed because no STR data are available for the Sardinian mouflon, and which could have been used to solve the forensic case described here. All 16 loci of the confiscated mouflon carcass were also genotyped.

The sheep and the mouflon are genetically very closely related, the latter considered the wild counterpart of the former. As mentioned above, no mitochondrial or single-locus markers capable of discriminating between the two subspecies are available. The source taxon of the carcass was thus identified through a full Bayesian assignment test, based on allele frequency data. We first tested loci independence and conformity to HWE; they are requisite for applying assignment tests, since violations could result in biased estimates of the probability of membership. Tests for deviations from linkage equilibrium comparing locus pairs across populations (10^4 iterations and 10^4 dememorizations as Markov chain length) were significant in eight (7%) of the 120 pairwise comparisons made, and following Bonferroni correction. Since no locus combinations were in linkage disequilibrium in both populations (all being restricted to the mouflon population) the entire set of loci was included in the subsequent analyses.

Bonferroni-corrected probability tests for departures from HWE showed significant heterozygote deficiency ($p < 0.001$) at three (INRA023, INRA172 and ILST087) and two (McM527 and TGLA53) loci in the mouflon and sheep populations, respectively; Cornuet et al. [35] showed that slight deviations from HWE (deficit of heterozygotes) have little effect in simulated assignment tests.

The estimated F_{ST} value between sheep and mouflon was 0.25 (0.19–0.31; 95% confidence interval, 1000 bootstraps); this shows the two populations to be sufficiently divergent to ensure a high level of certainty when the assignment test is applied to identify the source population of the sample examined. Results from simulations and from real data obtained by Manel et al. [36] showed that about 10 loci with an expected heterozygosity (H) of 0.60 and an F_{ST} value of 0.20 for two populations (30–50 individuals/population) provided nearly 100% assignment accuracy. Our results, in which 14 of 16 loci had $H > 0.60$, compare favourably with those obtained by Manel et al. [36].

Once all the prerequisites had been satisfied we applied the Bayesian approach to distinguish the populations and to assign the casework sample to its correct population. Using Structure, individual multilocus genotypes were grouped into K panmictic clusters. Setting K as unknown *a priori*, the highest posterior probability was obtained for $K = 2$ (values not provided), and indicates that two genetically distinct populations are represented in the full data set. The average proportional membership values (q) were 0.992 and 0.930 for the inferred sheep and mouflon clusters, respectively. To assign individuals we set the threshold probability at $q_i > 0.90$ for the following reason: mouflon can crossbreed with domestic sheep, aided by the fact that Sardinian farmers habitually allow their sheep to graze in the wild. Occasionally, crossbreds enter the mouflon population with resultant introgression of sheep alleles; the resulting admixed genotypes of dual ancestry can be randomly sampled from the mouflon populations. Sheep are not expected to have admixed genotypes, so the threshold $q_i > 0.90$ was selected as all sheep samples had been correctly assigned to their own species. According to the 90% probability intervals (PI) all individual sheep, except two, had a q_i value > than 0.90. According to their individual q_i values (>0.95) all 96 sheep could be assigned to their correct population. Of the 58 mouflon samples, 47 (81%) had q_i values > 0.93 and so could be assigned to their correct population; the remaining 11 samples, originally identified as mouflon in that they lacked sheep-like characters in the phenotype, revealed signs of crossbreeding as they had q_i values that ranged between 0.29 and 0.43, placing them (partly) within the sheep population. The casework carcass, with a $q_i > 0.99$, was assigned to the mouflon population, and demonstrated that the sheep breeder had been untruthful when he had claimed that the carcass belonged to that of one of his flock.

In spite of the relatively high percentage (19%) of admixed genotypes, the sheep and mouflon populations remained sharply separated (Fig. 2). Admixed genotypes might be second generation crossbreds or backcrosses as suggested using the programme Structure (data not provided). This may have diluted the

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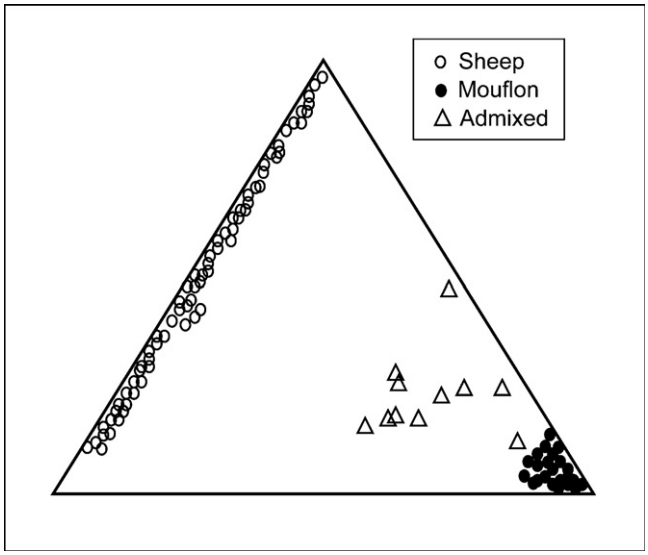

Fig. 2. Triangle plotting of the results from Structure when two populations are assumed in the dataset ($K = 2$), showing the separation of mouflon (black circles) and sheep (open circles). Admixed individuals are indicated by open triangles.

contribution of sheep alleles to the current mouflon gene pool, preventing homogenization of the two populations. As a result, the Bayesian approach remains adequate for assigning individuals to their correct population, facilitating the unambiguous identification of the mouflon carcass.

4.2. Matching casework samples

DNA was successfully recovered from all of the nine bloodstains that had been collected at the breeder's sheepfold. Complete 16-loci genotypes were obtained from all the samples, except in one where amplification failed at four loci. These blood profiles matched perfectly the profile of the carcass. Probabilities of identity in the mouflon population were calculated using the allele frequencies in the database; this included mixed genotypes due to the original accidental sampling of crossbred individuals that had a mouflon-like phenotype. A poacher could make the same error. For forensic purposes we chose to include the crossbred genotypes in the database. P_{ID} values were estimated at different relationship levels. The estimated probability of any two unrelated mouflon individuals by chance alone sharing the same multilocus genotype was about 1 in 22 million (Table 1), and decreased only slightly (to 1 in 20 million) when the estimated F_{IS} value of 0.19 was included, as can be expected in the case of multiallelic loci and moderately high single locus heterozygosity [31] and which formed part of the database. In practice, only values reflecting very close relationships will vary significantly from values for unrelated individuals, differing by one to four orders of magnitude [34]. As expected, the P_{ID} values for parent/offspring dyads decreased markedly (1 in 23,000) compared to the same value for unrelated individuals, dropping to 1 in 2300 for siblings. The population of Sardinian mouflon stands currently at about 3000 head; this makes it highly unlikely that any two individuals taken at random from this population would show the same 16-loci profile, even in the event of their being closely related.

Table 1
Values of average probability of identity (P_{ID}) for the mouflon population, and random match probability (RMP) for the casework sample, calculated for unrelated individuals, parent/offspring dyads, siblings and using the estimated inbreeding coefficient F_{IS} (0.19). Likelihood ratios are shown in parentheses.

	P_{ID}	RMP
Unrelated individuals	4.6×10^{-8} (1 in 22 million)	3.0×10^{-11} (1 in 33 billion)
F_{IS}	5.0×10^{-8} (1 in 20 million)	1.2×10^{-8} (1 in 80 million)
Parent/offspring	4.3×10^{-5} (1 in 23,000)	1.9×10^{-6} (1 in 526,000)
Siblings	4.4×10^{-4} (1 in 2300)	1.8×10^{-4} (1 in 5600)

The match probabilities for the casework profile (Table 1) were 1 in 33 billion for unrelated individuals, and 1 in 80 million when the actual F_{IS} value was included in the calculation. Under the sibling scenario the RMP was 1 in 5600. Thus, even when taking the very conservative upper bound into consideration, the evidence renders it 5600 times more likely that the bloodstains came from the carcass confiscated from the breeder, and not from another mouflon in the population. In consequence, the breeder's claim that the carcass in his truck belonged to that of one of his sheep was no longer tenable; accordingly, he was indicted for the illegal poaching of protected wildlife.

The results indicate our 16-loci profile system to have been sufficiently sensitive for identifying and matching individual Sardinian mouflon samples for forensic purposes.

5. Conclusions

Gene frequency databases are essential when the source population of individual samples of biological origin need to be identified molecularly. However, for an assignment test to succeed, the populations under scrutiny need to be genetically differentiated. Such population databases are very useful to wildlife forensics, especially when the assignment of forensic samples involves closely related taxa and no other taxon-specific markers are available. This study has shown that a suitable panel of short tandem repeat (STR) loci will facilitate reliable identification of a source species even when only small traces of evidence exist, in this instance nine bloodstains linked to the carcass of a Sardinian mouflon hunted illegally. Over the last two decades molecular forensics has come of age and is now being used increasingly to resolve many environmental crimes that include the illegal harvest of protected wildlife. Previously, many such crimes have proved difficult to resolve with the consequence that perpetrators, till now, have escaped prosecution.

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