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CASE REPORT

Identifying a hunter responsible for killing a hunting dog by individual-specific genetic profiling of wild boar DNA transferred to the canine during the accidental shooting

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Abstract While genetic profiling can be a powerful tool to solve wildlife crime, comparably few examples of individual identification in wildlife forensics are available in the literature. Here, we report a case of an accidental shooting of a hunting dog during a wild boar drive hunt. The market value of trained hunting dogs can reach several thousand euro. No one admitted to killing the dog. Wild boar hairs were found in the dog's wound, suggesting that the bullet first hit a wild boar and then the dog. Since it was known who harvested each boar, we aimed to use individual-specific genetic profiles to link these hairs to a bagged animal and to identify the culprit. We genotyped 19 harvested boar and the unknown hair sample using 13 STRs. In the case of the hair sample, we performed multiple genotyping to ensure the reliability of the genetic profile. We showed that we genotyped sufficient loci to distinguish between separate individuals with certainty. While the three most informative loci were enough to differentiate the 19 reference individuals, we did find a perfect match at all 13 STRs between the hair DNA and one tissue sample. Since our methods were reliable and reproducible, we passed the relevant information on to forestry officials who will use the information we have provided to attempt to find an amicable solution.

Keywords *Canis lupus familiaris* · Case study · Microsatellites · *Sus scofa* · Wildlife forensics · Validation

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Introduction

The usefulness of genetic profiling in identifying suspects in human forensic cases has furthered interest in developing wildlife forensic science as a tool in solving wildlife crime [1, 2]. Genetic markers can be of great use in the identification of illegally traded animal products, where species identification by morphological features is no longer possible. For instance, DNA sequences have been generated from commercially available whale and dolphin meat to identify illegally harvested species [3]. Similarly to human cases, individual-specific genetic profiling established using short tandem repeat (STRs) markers can provide a powerful tool for linking a suspect to a misdemeanor. For example, DNA profiling has been used to link blood stains on a suspect's knife to an illegally harvested wild boar [4]. However, while molecular genetic technologies are now routinely used for animal conservation studies, comparably few examples of the use of STR markers for individual identification in a wildlife forensic case are available in the literature [1, 4, 5] despite its common use in conservation genetics [6, 7].

Case report

A trained hunting dog (*Canis lupus familiaris*) was accidentally killed during a wild boar (*Sus scrofa*) drive hunt in central Germany on the 25th November 2015 between 10 a.m. and 1.30 p.m. The dog was found severely injured with bullet wounds 650 m away from the nearest posted hunter. It later succumbed to its injuries. The responsible hunter was invited to admit to the killing, since the dog's owner had a legal right to be financially compensated for his loss (the market value of trained hunting dogs can reach

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several thousand euro [8]). No one voluntarily acknowledged involvement. During an emergency operation on the dog the veterinarian found wild boar hairs in the dog's abdominal cavity, suggesting that the bullet first hit a wild boar and then the dog (Fig. 1). It was realized that genetic methods would allow these hairs to be linked to a specific wild boar and, since it was known who harvested each of the 19 bagged animals, in turn this information would make it possible to identify the person responsible for shooting the dog.

Laboratory work

The wild boar hairs collected from the wound and muscle samples from the 19 harvested boars were stored in 96 % absolute ethanol. DNA was extracted from tissue samples using an ammonium acetate-based salting-out procedure [9]. In order to ensure sufficient DNA quantity for reliable genotyping, six hair roots were pooled for DNA extraction using the MagJET^{TM} Genomic DNA kit (Thermo Fisher Scientific). DNA samples were quantified using a DropSense 16 (Trinean). All samples were genotyped using 13 STR loci (*S0005*, *S0026*, *S0090*, *S0097*, *S0115*, *S0226*, *SW112*, *SW240*, *SW632*, *SW857*, *SW911*, *SW936*, and *SW951*). While the use of STRs with tetranucleotide repeats might be preferable in forensic studies (dinucleotides suffer from increased stutter and variability in the relative height of the two peaks of a heterozygote), dinucleotide markers that are already widely used in animal genetic studies are acceptable [10]. The markers used here have been recommended by the Pig Genome Mapping Project for pig biodiversity studies, as they were highly polymorphic, not linked, and there was no indication of null alleles [11]. The loci have been used in a number of wild boar population genetic studies. There was no evidence of systematic deviations from Hardy–Weinberg and linkage disequilibria [12, 13]. Genotyping was performed using three multiplex polymerase chain reactions (PCR). Details on multiplex composition, PCR reaction times, and temperatures can be found in Frantz et al. [13]. PCR products were separated using an ABI 3730xl automated DNA sequencer (Applied Biosystems), and the data were analyzed using GeneMapper version 4.0 (Applied Biosystems).

In order to generate a reliable genetic profile from the hair DNA sample, we first analyzed three positive PCRs per STR locus. Loci were accepted as heterozygous if both alleles were observed at least twice. Loci were accepted as homozygous after a further four additional positive PCRs. Samples were defined as having identical genetic profiles if they shared the same genotypes at all 13 STR loci. After successfully matching the hair DNA sample with one of the 19 wild boar tissue samples, a new tissue DNA extraction from the suspect wild boar was performed to exclude the possibility of a pipetting or labeling error.

Data analysis

We used the 19 reference samples to test for the significance of heterozygote deficiency or excess with the Markov chain method in GENEPOP 3.4 [14] with 10,000 dememorization steps, 500 batches, and 10,000 subsequent iterations. Pairs of loci were tested for linkage disequilibrium using an exact test based on a Markov chain method

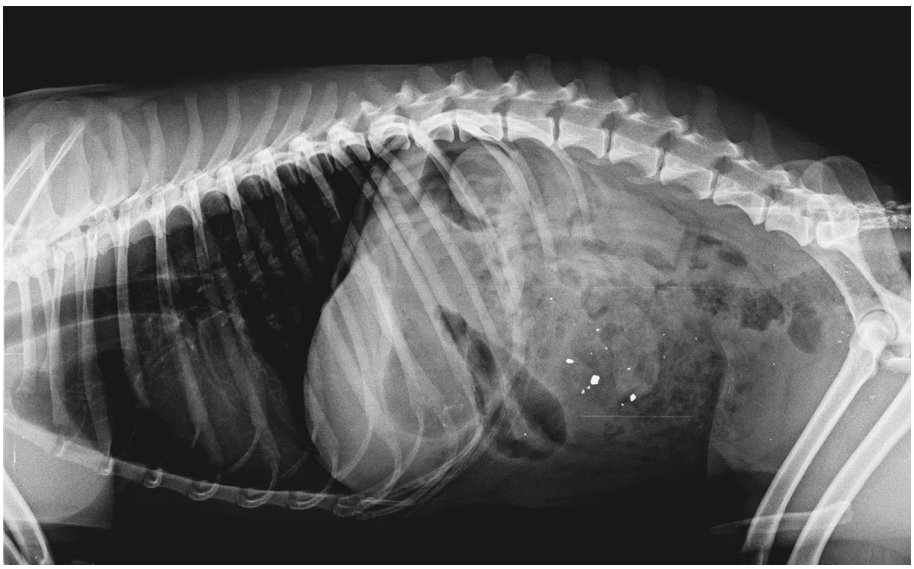

Fig. 1 X-ray showing the dog's bullet-related abdominal wounds

Table 1 Diversity statistics of the thirteen STR loci used in the present study

Locus	*A*	H_o	uH_e	HW P_{ID}	cum. HW P_{ID}	P_{ID_sib}	cum. P_{ID_sib}
S0005	9	0.947	0.900	0.029	2.90×10^{-2}	0.3195	0.3195
S0097	7	0.842	0.825	0.068	1.97×10^{-3}	0.3652	0.1167
S0090	5	0.895	0.737	0.127	2.50×10^{-4}	0.4231	0.0494
SW857	5	0.79	0.708	0.157	3.93×10^{-5}	0.4444	0.0219
SW112	4	0.737	0.647	0.201	7.90×10^{-6}	0.4851	0.0106
S0026	4	0.526	0.607	0.218	1.72×10^{-6}	0.5089	0.0054
SW936	4	0.631	0.585	0.230	3.96×10^{-7}	0.5215	0.0028
SW632	4	0.632	0.518	0.301	1.19×10^{-7}	0.5732	0.0016
S0115	3	0.526	0.494	0.345	4.11×10^{-8}	0.5960	0.0010
SW911	3	0.263	0.496	0.361	1.49×10^{-8}	0.5986	0.0006
S0226	3	0.526	0.482	0.340	5.05×10^{-9}	0.6003	0.0003
SW240	3	0.158	0.152	0.731	3.69×10^{-9}	0.8588	0.0003
SW951	1	0	0	1.000	3.69×10^{-9}	1.0000	0.0003

A, number of alleles; H_o, observed heterozygosity; uH_e, unbiased expected heterozygosity; HW P_{ID}, probability of identity calculated based on Hardy–Weinberg expectations; cum. HW P_{ID}, cumulative product of individual HW P_{ID} values; P_{ID_sib}, sibling probability of identify; cum. P_{ID_sib}, cumulative product of individual P_{ID_sib} values; Loci are arranged in order of decreasing individual P_{ID_sib} value. The estimated overall P_{ID} value corresponds to the cumulative P_{ID} value of all thirteen loci

as implemented in GENEPOP 3.4. The false discovery rate (FDR) technique was used to eliminate false assignment of significance by chance [15]. We used the 19 reference samples to calculate observed (H_O) and unbiased expected (uH_E) heterozygosities for each locus, using GENETIX 4.05.2 [16].

According to Waits et al. [17], in order to be applicable in the majority of law enforcement forensic applications in natural populations, genetic profiles should consist of enough STR loci to distinguish between individuals with between 99.9 and 99.99 % certainty. We assessed the probability that two individuals in a population share the same alleles at all 13 loci by chance by calculating a probability of identity (P_{ID}) statistic. This statistic is derived from allele frequencies, but assumes random associations between alleles within and among loci, and thus that they are in Hardy–Weinberg equilibrium. Because of the likely presence of related animals in the dataset, we also used an estimate of P_{ID} among siblings ($P_{ID\text{-}Sib}$; Waits et al. [17]), which provides the most conservative number of loci required to resolve all individuals. We used the program GENECAP [18] to calculate P_{ID} statistics based on the 19 harvested wild boars, arranging loci in order of decreasing value of single-locus $P_{ID\text{-}Sib}$. PROB-ID5 (G. Luikart, unpublished) was used to compute the proportion of all possible pairs of individuals that had identical genotypes. Finally, we used GENECAP to identify matching genotypes and to test for the presence of genetic profiles that only differed at one or two alleles.

Discussion

The hair DNA extract contained 23 ng/µl of double stranded DNA. We did not observe a single case of allelic dropout. All homozygote consensus genotypes were observed at least seven times. Locus *SW951* was monomorphic in the 19 reference individuals. While locus *SW911* deviated from Hardy–Weinberg equilibrium, this deviation was not significant after correcting for multiple tests ($P > 0.004$). While nine loci were in linkage disequilibrium before correcting for multiple test, one pair (*SW857* & *SW911*) was afterward ($P < 0.0008$). Omitting locus *SW951*, we observed three to nine alleles per locus, with H_o varying between 0.158 and 0.947 and unbiased H_e between 0.152 and 0.900 (Table 1).

While the estimated overall HW P_{ID} was 3.67×10^{-9} (and the four most informative loci gave a HW $P_{ID} < 0.0001$), the $P_{ID\text{-}sib}$ estimate suggested that there was a 2.98×10^{-4} chance of two individuals sharing an identical genetic profile by chance (Table 1). The three most informative loci were sufficient to distinguish between all the individuals in the reference dataset (Table 2). Finally, we found a perfect match between the hair DNA sample and one of the reference samples (Table 2). No profiles differed by one or two alleles only.

Given the forensic nature of the case and the possible financial consequences for the perpetrator, it was important to show that our analytical molecular methods were robust, reliable, and repeatable. We choose 13 STR loci that had previously been shown to be unlinked, with no indication of null alleles [11]. Even though we were able to pool

Table 2 Genetic profiles obtained from one hair DNA sample recovered from the dog and the 19 reference wild boar samples

Sample name	Alleles at microsatellite loci																									
	S0005		*S0097*		*S0090*		*SW857*		*SW112*		*S0026*		*SW936*		*SW632*		*S0115*		*SW911*		*S0226*		*SW240*		*SW951*	
Hair DNA	228	236	230	234	243	245	150	150	117	117	92	92	83	85	162	167	146	156	163	163	189	189	83	99	120	120
S5059	228	254	230	232	243	247	150	152	110	117	92	94	81	83	162	162	156	158	163	163	180	189	99	99	120	120
S5068	238	238	230	238	243	249	152	152	117	117	92	92	81	83	162	167	156	156	163	163	189	189	83	99	120	120
S5071	253	259	218	238	245	249	148	152	115	117	92	94	83	83	162	162	146	146	159	163	180	182	99	99	120	120
S5077	251	257	234	241	245	249	148	148	110	117	92	100	83	98	162	162	146	158	159	159	182	189	99	99	120	120
S5081	251	259	238	238	243	245	148	158	110	117	92	98	83	85	162	177	146	146	159	163	182	189	99	99	120	120
S5084	257	259	218	241	249	249	150	154	115	117	94	94	83	98	162	162	146	146	159	163	180	182	99	99	120	120
S5086	254	259	218	238	245	253	150	152	112	117	92	100	83	85	162	167	146	156	163	163	189	189	99	99	120	120
S6054	254	259	218	238	245	249	148	150	112	112	92	100	81	83	162	177	146	156	159	165	189	189	99	116	120	120
S6059	254	257	236	241	245	249	148	152	110	117	92	92	83	83	162	167	146	156	159	159	182	189	99	99	120	120
S6067	238	246	234	241	245	249	150	152	117	117	92	92	81	83	167	175	146	146	163	163	189	189	99	99	120	120
S6069	251	254	234	241	245	249	148	152	110	110	92	92	83	83	162	167	146	146	159	159	182	189	99	99	120	120
S6070	**228**	**236**	**230**	**234**	**243**	**245**	**150**	**150**	**117**	**117**	**92**	**92**	**83**	**85**	**162**	**167**	**146**	**156**	**163**	**163**	**189**	**189**	**83**	**99**	**120**	**120**
S6073	236	246	234	241	245	245	150	152	110	117	92	98	83	83	162	162	146	156	163	163	182	189	99	99	120	120
S6075	246	251	234	241	245	249	148	152	110	117	92	100	83	83	162	162	146	156	159	159	182	189	99	99	120	120
S6078	238	251	234	238	243	245	150	150	110	112	92	98	83	85	162	167	146	146	163	163	182	189	99	99	120	120
S6085	236	246	234	238	243	245	150	152	110	117	92	100	83	83	162	167	146	146	163	163	189	189	99	99	120	120
S7069	238	246	234	234	243	253	150	152	110	117	100	100	81	83	162	167	146	146	163	163	189	189	99	99	120	120
S7077	253	259	218	218	247	249	148	150	112	117	92	92	81	81	162	177	146	156	159	163	189	189	99	99	120	120
S7086	236	238	230	238	245	253	150	152	110	117	100	100	81	98	162	162	146	156	163	163	189	189	99	99	120	120

The hair sample represents a consensus genotyped obtained after repeated genotyping (no allelic dropout was observed). Loci are arranged in order of decreasing individual P_{ID_sib} value. The matching reference genotype is in bold

different hair samples in one DNA extraction, we nevertheless genotyped the hair DNA sample multiple times to avoid genotyping error. After establishing a perfect match, we re-extracted and re-genotyped the DNA sample in question to confidently exclude pipetting or labeling error. The fact that no profile differed by one or two alleles only suggested that there were no instances of allelic dropout. In summary, we took the precautions necessary to ensure that our results stood up to scrutiny. However, we used methodologies appropriate for working with good quality and quantity DNA samples. It is likely that methodologies more typically required for forensic (and non-invasive) genotyping would need to be employed for more difficult material.

Perhaps this study represents a special case, since the pool of possible candidates for a match was rather low (i.e., 19 individuals), but on the other hand, the boar originated from the same drive hunt and some individuals were therefore likely to be highly related. This is also the most likely explanation for the linkage disequilibrium that we observed with two loci that were physically unlinked. Nevertheless, we found that the three most informative loci were sufficient to generate genetic profiles that were specific for each of the 19 bagged animals and to identify the wild boar of interest. However, by genotyping 13 STRs altogether, we were able to distinguish between different individuals with 99.97 % certainty, falling well within the 99.9–99.99 % range postulated by Waits et al. [17]. Given that we observed a match with one of the 19 bagged animals, we can confidently exclude the possibility that the hairs originated from an animal not included in this group.

The probability of identity depends on the number and diversity of samples included in the study [19]. In this case, sample size was limited to the 19 wild boar shot during the given hunt, because it was assumed that one of them would lead to a match with the hair found in the dog. While the results were validated for the presented study case, they may not hold true beyond the study area due to differences in allele frequencies and relatedness among individuals in other wild boar populations. It is advised that probabilities of identity are newly assessed for different populations.

Here, the case of who accidentally shot a dog during a drive hunt was resolved using STR-based individual specific genetic profiling to link the shooting of the dog to that of a wild boar, and thereby identify the culprit. We passed the relevant information on to forestry officials who will use it to attempt to find an amicable solution.

Key Points

1. While genetic profiling can be a powerful tool to solve wildlife crime, comparably few examples of individual identification in wildlife forensics are available in the literature.
2. In this study we aimed to use STRs to link wild boar hairs found in the bullet wounds of a valuable hunting dog accidentally killed during a drive hunt to the wild boar which had first been killed by the bullet.
3. We genotyped the hair sample multiple times to ensure a reliable genetic profile and genetic profiles of reference individuals showed that we genotyped sufficient loci to distinguish between separate individuals with certainty.
4. Because our results were reliable and repeatable and who harvested each wild boar was known, it was possible to identify the hunter that was responsible for killing both animals.

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