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SHORT COMMUNICATION

Wolf or dog? Genetic identification of predators from saliva collected around bite wounds on prey

Anna-Karin Sundqvist · Hans Ellegren · Carles Vilà

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Abstract Wolf predation on livestock is a management problem in many areas and is often used to justify control measures against the wolves. However, wolves coexist with dogs across their range, and dogs could be responsible for attacks blamed on wolves. In this study we evaluate the possibility of obtaining sufficient DNA for species identification of the predator from saliva remaining close to bite wounds following a canid attack. Predator DNA of reasonably high quality was successfully extracted from bite wounds on two sheep that had been attacked on a farm and were genotyped using six informative microsatellite markers. A single consensus genotype could be constructed from the bite wounds of both sheep which we compared to genotypes obtained from Scandinavian wolves and dogs. The results clearly showed that the saliva sampled originated from a single dog. This report thus demonstrates the feasibility of predator species identification from bite wounds and also illustrates that it can not be taken for granted that wolves are responsible for canid livestock kills.

Keywords Predation · Wolves · Microsatellites · Non-invasive genotyping

Introduction

Gray wolf (*Canis lupus*) predation on livestock is a management problem in many areas over the world. For instance, predation is often used to justify increased control measures against wolves, including "exclusion areas" where wolves are not allowed or where the population growth should be limited (Ministry of Agriculture and Forestry 2005). In addition, livestock attacks can promote a negative public attitude towards wolves (Ericsson and Heberlein 2003; Chavez et al. 2005), hindering any management program that includes promoting the long term survival of wolf populations. However, other predators also kill livestock. Since the predator is rarely seen, in most cases direct observation is not a useful method to identify the species responsible for an attack. Instead, traces left on the prey site (tracks, hair, blood, condition of the surroundings, etc) as well as bite marks on the prey are usually used for identification of the predator species involved. While this can be done with some confidence for at least some predators, it is more difficult in other cases. Importantly, wolves coexist with domestic dogs (*Canis familiaris*) across most of their range, and dogs can also be responsible for livestock attacks. Although evidence left at the site of an attack usually differs between wolves and dogs because wolves are more skilful hunters, the identification of the culprit is not always clear. A more reliable method to distinguish between livestock attacks conducted by wolves or by dogs is necessary. First, if wolves are blamed for attacks they are not responsible for, wolf conservation and management can be hampered. Second, the correct identification of the predator may also be of economic interest because in many areas farmers get compensation for their losses if their livestock were attacked by wolves, but not if attacked by dogs.

It has previously been shown that it is possible to successfully amplify predator DNA from saliva collected from bite wounds. Blejwas et al. (2006) and Williams et al. (2003) were able to amplify mitochondrial DNA and a few nuclear loci from wounds on sheep to show that coyotes

A.-K. Sundqvist · H. Ellegren · C. Vilà (✉)
Department of Evolutionary Biology, Uppsala University, SE-752 36 Uppsala, Sweden
e-mail: carles.vila@ebc.uu.se

(*Canis latrans*) were responsible for the attacks. However, since different predators use different killing techniques and might leave different amounts of saliva not mixed with the prey's blood, it is not known if this approach can be broadly used. Further, since dogs and wolves have diverged recently (Vilà et al. 1997), mitochondrial DNA alone may not suffice to differentiate the two species. Also, since dogs and wolves can hybridize in the wild (as observed in Scandinavia, Latvia and Italy; Vilà et al. 2003; Andersone et al. 2002; Randi and Lucchini 2002; Verardi et al. 2006), a method that allows identification not only of wolves and dogs but also hybrids is needed. In this study we evaluate the possibility of obtaining DNA from saliva around bite wounds of high enough quality to genetically distinguish wolves and dogs. We use a panel of biparentlly inherited canine microsatellite markers that have been shown in previous studies to differentiate dogs, wolves and their hybrids (Vilà et al. 2003).

Material and methods

Samples were collected in central Sweden from bite wounds on two sheep that had been seriously injured in a single canid attack. One sample per wound was collected, five from one sheep (sample 0005) and three from the other (3016). The samples were taken with cotton swabs 6–10 h after the attack from the edges of bite wounds, carefully trying to avoid getting sheep blood. The swabs were immediately put into 15 ml plastic tubes and let dry at room temperature. The next day they were put in a freezer and kept there until processing. Blood samples were also taken from the sheep that had been attacked and mouth swabs from the two herding dogs in the farm.

One day before DNA extraction, the cotton swabs were put in 1 ml Laird's buffer (0.1 M Tris–HCl, 5 mM EDTA, 0.2 M NaCl, 7 mM SDS, adjusted to pH 8.5) and left at room temperature overnight. Genomic DNA was extracted from 400 µl of the buffer. The cells were digested overnight with 0.3 mg of proteinase K at 37°C. DNA was then extracted using a modified phenol/chloroform protocol (Sambrook et al. 1989). Eight autosomal microsatellites were amplified from all samples: *2096* (Francisco et al. 1996), *u109*, *u173*, *u225*, *u250* (Ostrander et al. 1993), *vWF* (Shibuya et al. 1994), *PEZ03* and *PEZ05* (Perkin-Elmer, Zoogen; see NHGRI Dog Genome Project at http://research.nhgri.nih.gov/dog_genome/). The same PCR mix and PCR profile was used for all eight markers. The PCR mix included 1X PCR buffer (Qiagen), 3.5 mM $MgCl_2$, 0.25 mM dNTP, 0.32 µM of each primer, 0.025X Q solution, 0.5 U Hot Star Taq polymerase (Qiagen) and 2 µl of DNA template. The PCR profile included an initial denaturation step at 95°C for 15 min followed by 12 touchdown cycles (30 s at 95°C, 30 s starting at 58°C and decreasing 0.5°C each cycle and 60 s at 72°C), followed by 30 additional cycles (95°C for 30 s, 52°C for 30 s and 72°C for 60 s), and a final extension step at 72°C for 10 min. PCR products were pooled in three different groups (u109, u173 and u225), (PEZ05, u250 and vWF) and (PEZ03 and 2096) and electrophoresed on a MegaBACE 1000™ instrument (Amersham Biosciences). Genotypes were scored using the software Genetic Profiler v2.2 (Amersham Biosciences). Three replicates were run for every sample and marker, since allelic dropouts are a common problem when working with low quality DNA samples (Taberlet et al. 1996). For a single-locus genotype to be considered as reliable two replicates producing identical genotypes were required for heterozygotes and three replicates for homozygote, following the criteria of Hedmark and Ellegren (2006). The likelihood of finding other individual dogs and wolves with the same genotype was assessed by the probability of identity (Paetkau and Strobeck 1994). These calculations were based on the allelic frequencies estimated for Scandinavian wolves and dogs (Vilà et al. 2003). A visual representation of the similarity between the obtained genotypes and those in dogs and wolves was generated using a factorial correspondence analysis (FCA) in GENETIX 4.05 (Belkhir et al. 1996–2004).

Results and discussion

Two of the markers tested, *u250* and *2096*, amplified fragments of similar length to those in canids in the two sheep blood samples. The stutter bands produced by these amplifications were often different from those produced by canid samples, and the poor amplification led to inconsistent results (data not shown). However, to avoid any possible mistyping, these markers were excluded from further analyses. This cross-amplification implies that results of non-invasive wolf genotyping could be affected by the amplification of DNA from prey items and cross-amplification experiments should be implemented when possible. The other six markers did not amplify sheep DNA.

There were significant differences in genotyping success between the samples (0–83%). For one sample no amplification was successful (3016-3). Three of the samples yielded poor results (0005-2, 0005-3 and 3016-2); although occasional amplification was seen, these were not enough to build consensus genotypes at any locus. However, for the remaining four samples it was possible to obtain reliable consensus genotypes for one to four microsatellite loci. For one of these samples, 0005-4, a microsatellite genotype could be scored in 15 out of 18 amplifications.

Table 1 Samples analyzed and genotypes obtained from each replicate

	vWF	vWF	pez05	pez05	pez03	pez03	u109	u109	u173	u173	u225	u225	
0005-1 rep1	-	-	104	104	124	124	148	150	105	105	164	168	
0005-1 rep2	-	-	104	104	-	-	148	150	105	105	164	168	
0005-1 rep3	-	-	104	104	-	-	-	-	-	-	-	-	
0005-1 cons.			**104**	**104**			**148**	**150**			**164**	**168**	
0005-2 rep1	164	164	104	104	-	-	148	148	-	-	-	-	
0005-2 rep2	-	-	104	104	-	-	-	-	-	-	-	-	
0005-2 rep3	-	-	-	-	-	-	-	-	-	-	-	-	
0005-2 cons.													
0005-3 rep1	-	-	104	104	-	-	148	148	-	-	-	-	
0005-3 rep2	-	-	-	-	-	-	-	-	-	-	-	-	
0005-3 rep3	-	-	-	-	-	-	-	-	-	-	-	-	
0005-3 cons.													
0005-4 rep1	164	188	104	104	124	124	148	150	111	111	164	164	
0005-4 rep2	-	-	104	104	124	124	148	150	111	111	164	168	
0005-4 rep3	-	-	104	104	124	124	-	-	105	105	168	168	
0005-4 cons.			**104**	**104**	**124**	**124**	**148**	**150**			**164**	**168**	
0005-5 rep1	164	188	104	104	124	124	148	150	105	111	164	168	
0005-5 rep2	-	-	96	104	124	124	148	148	105	111	164	168	
0005-5 rep3	-	-	-	-	-	-	148	148	-	-	164	168	
0005-5 cons.									**105**	**111**	**164**	**168**	
3016-1 rep1	-	-	104	104	124	124	148	150	105	105	164	164	
3016-1 rep2	-	-	104	104	124	124	148	150	105	111	164	168	
3016-1 rep3	-	-	-	-	-	-	-	-	105	111	164	168	
3016-1 cons.							**148**	**150**	**105**	**111**	**164**	**168**	
3016-2 rep1	-	-	-	-	124	124	-	-	105	105	-	-	
3016-2 rep2	-	-	-	-	-	-	-	-	105	105	-	-	
3016-2 rep3	-	-	-	-	-	-	-	-	105	105	-	-	
3016-2 cons.									**105**	**105**			
3016-3 rep1	-	-	-	-	-	-	-	-	-	-	-	-	
3016-3 rep2	-	-	-	-	-	-	-	-	-	-	-	-	
3016-3 rep3	-	-	-	-	-	-	-	-	-	-	-	-	
3016-3 cons.													
Consensus	164	188	104	104	124	124	148	150	105	111	164	168	Combined Pid
Pid (dogs)	0.207		0.566		0.459		0.183		0.070		0.237		0.00016
Pid (wolves)	0.090		0.328		0.164		0.499		0.443		0.319		0.00034

Cases of allelic drop-outs are within boxes. Consensus genotypes are in bold. One false consensus genotype (see Results and discussion) is also marked with a box. One false allele is marked with a grey box. Probability of identity (Pid) for each marker and combining all markers is indicated for dogs (n = 85) and wolves (n = 192)

These results suggest that it is advisable to take multiple samples from an attack to ensure that at least some of them will provide sufficient amounts of predator DNA for microsatellite analysis. Since our aim was to assess the degree of success obtained with the different samples under a standard protocol (three replicates per sample) we did not make any effort to improve the genotyping success by further increasing the number of replicates for any sample. However, had we done so, we probably could have obtained complete genotypes, at least for samples 0005-1, 0005-4, 0005-5 and 3016-1.

Amplification success also varied between markers, from three to 13 out of 24, successful attempts for each marker (13–54%). The rate of allelic dropout between markers was also variable (27–69%). Dropout could be identified by the occasional amplification of just one allele in a heterozygous locus. The observation that the genotypes were consistent across all samples (see below) facilitated the identification of such cases. All together, 17 cases (45% of the amplifications of heterozygous loci) of allelic dropout were observed across all loci and samples (Table 1). These observations mirror what has been seen in other microsatellite studies using low-copy number DNA (e.g. Hedmark and Ellegren 2006; Björnerfeldt and Vilà 2007).

More then 2 alleles were not observed at any locus across all samples. Further, the genotypes obtained that fulfilled the criteria for reliability were consistent from sample to sample (except in one case), and were identical for the wounds from the two sheep (Table 1). In addition, the low probability of identity for the studied loci in dogs (Table 1) suggests that it would be extremely unlikely to find multiple dogs with the same genotype at multiple loci (although it would be more likely for wolves due to low diversity of this population). At one locus (*u173*), one consensus genotype (105/105 in sample 3016-2) differed from the consensus in two other samples (105/111 in 0005-5 and 3016-1), a discrepancy that can be explained by allelic drop-outs in sample 3016-2. Indeed, with a dropout rate of 45%, even three replicates shall in some cases be insufficient to identify a heterozygote. Out of 13 successful amplifications for locus *PEZ05*, 12 were homozygote (104, 104) and one heterozygote (96, 104). Since false alleles are known to occasionally occur when working with low quality DNA samples (Taberlet et al. 1996; Hedmark and Ellegren 2006), we consider allele 96 to be a false allele, rather then having 12 cases of allelic dropout. These observations, together with the fact that the two animals were injured—and not killed—in a single attack, suggest that there was a single predator involved.

We constructed a combined consensus genotype for the six investigated microsatellite loci for this individual and compared it to genotypes obtained from Scandinavian wolves and dogs contained in an internal database (see e.g. Vilà et al. 2003) and the two dogs from the farm where the attack took place. The genotype derived from the bite wounds fit well with the diversity observed within Scandinavian dogs and was well separated from all of more than 100 Scandinavian wolf samples in a FCA analysis (Fig. 1). The six markers used were basically enough to separate Scandinavian wolves and dogs. However, if more markers had been used, a clearer separation would have been obtained. Analysis with more markers show that the individuals with intermediate genotypes in Fig. 1 are not hybrids, in agreement with the observation that hybrids are uncommon (Vilà et al. 2003). To confirm that the result was not due to the construction of a consensus genotype by mixing different samples, we performed the same analysis using only the 4-loci genotype obtained from sample 0005-4. The FCA in this case still clustered this genotype perfectly within dogs and separated from wolves.

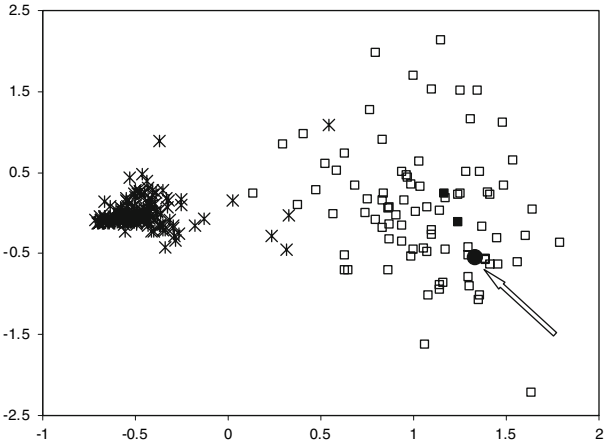

Fig. 1 Factorial correspondence analysis of wolves (stars), dogs (open squares), two farm dogs (filled squares) and the saliva sample (filled circle, marked with an arrow)

To make sure that the sheep had not been "contaminated" with dog DNA before the attack, genotypes from two herding dogs belonging to the sheep farm in question were also analyzed (data not shown). These two genotypes differed from the saliva samples with seven and nine alleles at the six microsatellite loci. Consequently, the herding dogs can be excluded as predators or "contaminators" of the attacked sheep. In the end, it is not known which individual was the predator but our analysis clearly shows that is was a dog, although not one of the two dogs from the farm.

These results confirm that it is possible to identify the predator by sampling saliva that is left in connection to bite wounds (Williams et al. 2003; Blejwas et al. 2006). Furthermore, this study shows that it is possible to obtain nuclear DNA of high enough quality to be able to distinguish between two closely related species, wolves and

dogs. Nevertheless, the rate of dropout was high and reliable identification may require the collection of multiple samples per wound. Additionally, a large number of replicates should be used for each marker.

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