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A coyote in sheep's clothing: predator identification from saliva

Christen Lenney Williams, Karen Blejwas, John J. Johnston, and Michael M. Jaeger

Abstract We used polymerase chain reaction (PCR)-based RFLP (restriction fragment length polymorphism) and microsatellite analyses to identify canid species, gender, and individual genotype in samples containing a large excess of domestic sheep DNA. These methods were then used to investigate the feasibility of identifying predators from saliva on predation wounds. We analyzed predation wound samples from 19 sheep carcasses. Coyote DNA was identified in 18 samples (95%), of which 17 contained male coyote DNA (94%) and 11 (61%) yielded heterozygous microsatellite genotypes at ≥ 1 locus. These methods have promise for genetic identification of individual predators.

Key words coyote, individual identification, microsatellite, non-invasive, saliva, sex identification, species identification, Sry, wildlife forensics

Minute amounts of unintentionally deposited DNA are now widely used in human forensics and criminal investigations. Specifically, DNA in saliva on stamps, envelopes, and even food items has been successfully genotyped and matched to suspects (Allen et al. 1994, Sweet and Hildebrand 1999). However, despite increasing use of non-invasive sample types like feces (Kohn et al. 1999, Ernest et al. 2000) and hair (Woods et al. 1999) in wildlife investigations and wide use of saliva in human investigations, saliva remains untapped as a DNA source for wildlife studies. Predation research is one obvious arena that could benefit from advances in using saliva as a DNA source.

Predation wounds potentially contain DNA from both prey (in the form of blood) and predator (in the form of saliva), and samples from such wounds could provide valuable information. Genetic iden-

tification of an individual predator from a predation wound sample has not previously been reported. Co-occurring DNA potentially raises problems for genetic analyses. Our goals were to identify genetic methods to investigate coyote (*Canis latrans*) predation on sheep and determine whether sheep DNA would interfere with the ability to correctly identify a coyote predator. Species identification relies on mitochondrial DNA present in high copy number DNA (Pilgrim et al. 1998), so we anticipated that sheep DNA should not interfere with the ability to identify coyote DNA in known mixed samples, unless there were overlapping restriction fragment patterns between predator and prey species. And even though the volume of predator saliva on a wound may be small, predator species identification from saliva on wounds should be feasible. However, gender determination in mixed-

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species samples may be problematic. Amplification of X- and Y-linked zinc finger protein (ZFX and ZFY) or the sex-determining Y (Sry) gene regions are routinely used to sex unknown individuals (Aasen and Medrano 1990, Amstrup et al. 1993, Taberlet et al. 1993, García-Muro et al. 1997, Kohn et al. 1999). However, ZFX/ZFY restriction fragment length polymorphism (RFLP) patterns of males or females (although different between genders) may be indistinguishable between different species. Additionally, whether sheep DNA regions would amplify with Sry primers developed in canids was not known. We therefore assessed what impact the presence of sheep DNA had on our ability to determine coyote gender in known mixed samples using both ZFX/ZFY and Sry approaches. Because microsatellite primers can give cross-species amplification, we also needed to determine whether sheep DNA interfered with coyote DNA microsatellite amplifications. We analyzed known mixed-species templates for species, gender, and individual microsatellite locus genotype, then analyzed samples taken from predation wounds on 19 sheep carcasses.

Materials and methods

Species determination

Coyote and sheep DNA was isolated from blood or tissue (DNeasy tissue kit, Qiagen, Valencia, Calif.) and quantified by flourometry (DyNA Quant 200, Hoefer Pharmacia Biotech Inc., San Francisco, Calif.). Coyote samples were from a previous study (Williams et al. 2003); sheep samples were from animals at National Wildlife Research Center facilities. We investigated the utility of canid-specific mitochondrial primers (Pilgrim et al. 1998) for determining presence of coyote DNA in mixed coyote:sheep DNA samples (Table 1). PCR amplification conditions were as in Pilgrim et al. (1998), with a 50°C annealing temperature for 40 cycles. For the geographical region from which we investigated predation events, we particularly required a reliable method to differentiate coyote predation from domestic dog (*Canis familiaris*) predation. Incomplete digestion of the dog mitochondrial DNA fragment using MvaI (Boehringer Mannheim, Roche Diagnostics Corporation, Indianapolis, Ind.) resulted in our inability to reliably distinguish coyotes from dogs with that restriction enzyme (not shown, cf. Pilgrim et al. 1998, Kohn et al. 1999). Instead, we evaluated the performance of a coyote-

specific Hinf I RFLP for species identification. This RFLP was identified by comparison of dog and coyote sequences deposited in GENBank (accession # U03575 and AF020700), as well as DNA sequence and RFLP data we generated in both species (not shown). Hinf I digestion resulted in fragments of 81 basepair (bp) and 76 bp in coyotes and fragments of 76 bp, 44 bp and 36 bp in dogs. Following mitochondrial amplification, PCR products were digested with Hinf I (Promega, Madison, Wisc.) at 37°C for 3 hours and electrophoresed through 3% NuSieve (BMA, Cambrex, East Rutherford, N.J.) containing 0.25 µg/ml ethidium bromide. Coyotes had 2 fragments at about 80 bp, but dogs had 1 (the 36 bp and 44 bp dog HinfI fragments were not easily visualized).

Gender determination

We compared 2 approaches to determining coyote gender in mixed-species samples. In the first approach, coyote ZFX and ZFY gene sequences

Table 1. Sensitivity of genetic analyses to determine coyote species, sex, and microsatellite genotype in mixed-species samples. Methods are as described. Template DNA for sex and microsatellite analyses included either male or female coyote and either male or female sheep DNA. Template DNA for species identification was performed with single sexes of each species. "Y" indicates coyote species, sex, or microsatellite genotype was correctly determined; "N" indicates coyote species, sex, or microsatellite genotype could not be determined. "-" indicates the analysis was not performed.

Coyote:sheep DNA	Species identification	Sexing		Microsatellite genotyping
		ZFX/ZFY	canid Sry	
2:1 (40ng:20ng)	-	Y	Y	-
1:1 (20ng:20ng)	-	Y	Y	-
1:1 (10ng:10ng)	-	Y	Y	-
1:2 (10ng:20ng)	-	Y	Y	-
1:2 (5ng:10ng)	-	Y	Y	-
1:10 (5ng:50ng)	-	N	Y	-
1:20 (2.5ng:50ng)	-	N	Y	-
1:10 (1ng:10ng)	-	N	Y	-
1:30 (1ng:30ng)	-	N	Y	-
1:90 (1ng:90ng)	-	N	Y	-
1:180 (0.5ng:90ng)	Y	-	Y	Y
1:360 (250pg:90ng)	Y	-	Y	Y
1:720 (125pg:90ng)	Y	-	Y	Y
1:1440 (62.5pg:90ng)	Y	-	Y	Y
1:14,400 (6.25pg:90ng)	Y	-	Y*	Y†

* 3 of 4 samples had microsatellite amplification, in the fourth microsatellite amplification failed, but Sry amplification was successful (Figure 1b, lane 25).

† Allelic drop-out did occur.

were determined using the primers P15EZ and P23EZ (García-Muro et al. 1997). PCR products were bidirectionally sequenced (Big Dye Terminator v3 Cycle Sequencing Kit, Applied Biosystems, Foster City, Calif.). Sequence data was collected using an automated DNA sequencer (ABI377 Prism, Applied Biosystems) and analyzed using DNA Sequence Analysis Software v3.4.1 (Applied Biosystems) and Sequencher v3.0 (Gene Codes Corporation, Ann Arbor, Mich.). Coyote ZFX sequence was determined by sequencing female coyote DNA. Coyote ZFY sequence was deduced by sequencing male coyote DNA (ZFX/ZFY) and subtracting the ZFX base at heterozygous sites. The coyote ZFX and ZFY sequences (deposited in GenBank; accession numbers AY145847 and AY145848) were compared with sheep ZFX and ZFY sequences (GenBank accession AJ000269, AJ000270) and aligned manually. The coyote ZFX possessed a unique Bmrl recognition site that was absent from coyote and sheep ZFY and sheep ZFX. Similarly, HaeIII RFLPs have been identified in dogs (García-Muro et al. 1997) that distinguish individual male from female coyotes (not shown) and, by comparison of coyote and sheep sequences, HaeIII was determined to be useful in identifying the presence of coyote ZFY in mixed sheep-coyote samples (both sheep ZFX and ZFY lack the HaeIII recognition site). ZFX/ZFY products were digested with 2U of either Bmrl (New England Biolabs, Beverly, Mass.) or HaeIII (Promega) at 37°C for 2 hours. We visualized the resulting fragments following electrophoresis through 2% NuSieve (Cambrex) containing 0.25 µg/ml ethidium bromide.

In the second approach to gender determination, we amplified the male-specific 104-bp Sry fragment using canine-specific primers (Meyers-Wallen et al. 1995). A canine microsatellite locus FH2010 (at which coyotes in this population have alleles in the range 229-237 bp; Williams et al. 2003) was co-amplified as an internal control to differentiate female coyotes from males that failed to amplify. One FH2010 primer was fluorescently labeled. Amplifications contained 267-µM Sry primers, 267-µM FH2010 primers, 1.5 mM MgCl₂, 0.1 µg/µl BSA, 1X AmpliTaq Gold buffer, and 0.5 units AmpliTaq Gold, (Applied Biosystems). The cycling profile was 95°C, 7' then 45 cycles of 94°C, 1', 53°C 1', 72°C 1', followed by 2' at 72°C and reactions were then held at 4°C. Sry/FH2010 amplifications did not require digestion prior to electrophoresis (for detection of the Sry fragment) and also were analyzed on 6%

Long Ranger Singel (BMA, Cambrex) using a fluorescent internal size standard and an automated DNA sequencer (ABI377 Prism, Applied Biosystems). The FH2010 alleles were analyzed using Genescan ver. 3.2.1 and Genotyper ver. 2.5 (Applied Biosystems).

To compare sensitivity of Sry and ZFX/ZFY analysis in mixed samples, templates of either DNA from individual coyote or sheep of known sex or a mixed-species sample were amplified for ZFX/ZFY and Sry regions. Ratios of coyote:sheep DNA in mixed-species amplifications ranged from 2:1 to 1:14,400 (Table 1).

Field samples

We used dry sterile cotton swabs to collect samples from lethal predation wounds on sheep during 1998 from a study site in northern California that had high levels of coyote predation (Blejwas et al. 2002). We surveyed the sheep pastures regularly and sampled carcasses if they were thought to be < 24 hours old. We analyzed one swab from each of 19 separate carcasses and in all instances, based on field necropsy, attributed predation to coyotes. These 19 swabs were all that were available for these genetic analyses that year (additional swabs had been collected but were unavailable). There were many punctures on most carcasses. To avoid collecting saliva from scavengers, we skinned the area around the punctures and took swabs only from puncture wounds that were identified as attack wounds, based on the presence of sub-dermal hemorrhaging. We individually air-dried the swabs for 24 hours, sealed swabs in a paper envelope, then placed them in a paper bag and froze them at 20°C until analyzed. We performed DNA extractions using a QIAamp DNA minikit (Qiagen) and the manufacturer's swab protocol with a 100-µl final elution. We used facilities and equipment dedicated to non-invasive samples for swab DNA handling, and aerosol-resistant tips (VWR, West Chester, Pa.). We used the methods described above for known samples for DNA amplification and analysis for species and gender identification. We selected 2 additional canid microsatellite loci, based on high levels of heterozygosity and allelic diversity in the coyote population in the area and reliability of scoring (FH2159 and FH2137, Williams et al. 2003). When genotyping samples that contain low amounts of template DNA, a multiple-tubes approach is recommended in which multiple amplifications are carried out independently

(Taberlet et al. 1996). We performed amplifications for each locus in quadruplicate, using conditions previously described (Williams et al. 2003) and a 45°C annealing temperature and 47 cycles. We analyzed an aliquot of each microsatellite amplification on 6% Long Ranger Singel (BMA, Cambrex) using a fluorescent internal size standard and an automated DNA sequencer (ABI377 Prism, Applied Biosystems). We analyzed genotypes using Genescan ver. 3.2.1 and Genotyper ver. 2.5 (Applied Biosystems). We scored only alleles detected at least twice, over all 4 amplifications.

Results

Analysis of known mixed-species samples

We readily distinguished coyotes from dogs using a coyote-specific *Hinf*I site (Figure 1a). Although sheep DNA yielded nonspecific amplification, following *Hinf*I digestion there were no sheep fragments in the region of interest (Figure 1a). Although felid DNA did amplify with these “canid-specific” mitochondrial primers, we easily recognized felids by the presence of larger, multiple fragments following amplification (heteroplasmy) and the absence of fragments in the region of interest following *Hinf*I digestion (Figure 1a). Other species also were readily identified (Figure 1a).

For gender identification, ZFX and ZFY amplification products co-migrated at about 450 bp for each species (not shown). A 145-bp *Bm*rI fragment reliably identified the presence of coyote ZFX and was detected in samples of coyote:sheep DNA ranging from 1:1 to 1:2 and down to 5 ng coyote DNA (not shown), but coyote or sheep ZFY and

sheep ZFX remained undigested. No *Hae*III recognition site exists within coyote ZFY, but *Hae*III cleaves 45 bases off coyote and sheep ZFX and sheep ZFY. Hence, all samples possess fragments at 45 bp and 402 bp, but we only observed a 447-bp fragment in samples that contained coyote ZFY and also in samples of coyote:sheep DNA ranging from 1:1 to 1:2 and down to 5 ng coyote DNA (not shown). Using the species-specific sex-linked ZFX/ZFY *Bm*rI and *Hae*III RFLPs, we were able to identify the presence of coyote ZFX and/or ZFY in samples that included twice as much sheep as coyote DNA (not shown). However, we detected neither coyote ZFX- or ZFY-specific *Bm*rI or *Hae*III fragments in samples with coyote:sheep DNA ratios less than 1:2 (Table 1), so we could not determine coyote gender in those samples using that approach.

In contrast, we correctly identified coyote gender over the entire range of dilutions using canid-specific *Sry* primers (Meyers-Wallen et al. 1995) in con-

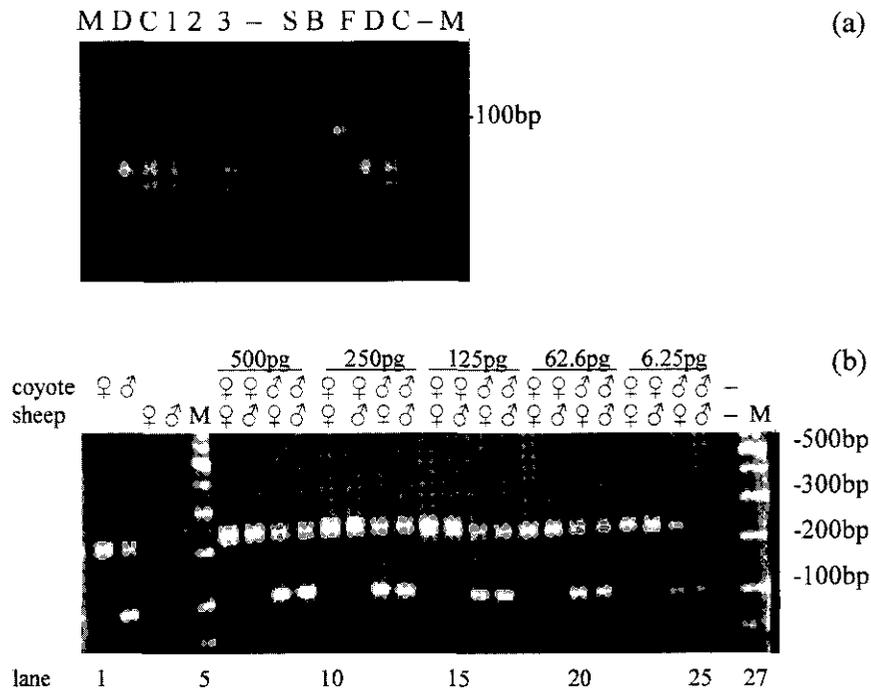


Figure 1. Species and sex identification. (a) Species identification: *Hinf*I digest of the canid-specific mitochondrial control region from single species. The species of each known template DNA is indicated above each lane: dog (*Canis familiaris*) D, coyote (*Canis latrans*) C, sheep (*Ovis aries*) S, gray fox (*Urocyon cinereoargenteus*) F, bobcat (*Lynx rufus*) B. Lanes labeled 1, 2, 3 are swab samples. (b) Sex identification: *Sry*/*FH2010* analysis of combined coyote and sheep template DNA. The template DNA for samples on the right portion of the gel contained 90ng sheep DNA and the amount of coyote DNA indicated above the gel. The sexes of the coyote and sheep are given for each sample. As positive controls, 5 ng of template DNA from only one species, as indicated, was used for the 4 samples on the left portion of the gel. For both panels, lanes labeled “-” are negative controls, and those labeled M contain a molecular size standard (100 bp DNA ladder, Promega, Madison, Wisc.), with fragment sizes indicated.

junction with a canid microsatellite locus. Results for known coyote: sheep DNA ratios of 1:180 to 1:14,400 are in Figure 1b. The presence of faint nonspecific amplification in the sample containing only sheep DNA (Figure 1b, lanes 3 and 4) did not interfere with the identification of either male or female coyote gender, including down to 6.25 pg coyote DNA (or the diploid content of about one coyote cell; Taberlet et al. 1996) in 90 ng sheep DNA (Figure 1b, lane 22-25). For one sample containing 6.25 pg coyote DNA, the Sry locus amplified but the microsatellite locus did not (Figure 1b, lane 25).

For microsatellite genotyping, we obtained the correct FH2010 microsatellite genotype from samples containing as little as 62.5 pg coyote DNA in 90 ng sheep DNA (1:1440). Below that level of coyote template DNA, the microsatellite locus amplified well enough to serve as a positive control, and we identified the correct gender. However, we detected allelic drop-out, indicating the appropriateness of a multiple-tubes approach (Taberlet et al. 1996) if coyote microsatellite genotypes are being determined.

Analysis of field samples

Eighteen of 19 swabs contained the diagnostic coyote mitochondrial RFLP (Figure 1a), and one appeared to be a dog. Of the 18 swabs identified as coyote, 17 (94%) contained the male-specific canid Sry fragment. One sample genotyped as a female when FH2010 and Sry were co-amplified, but genotyped as a male in a previous amplification of Sry alone (not shown). Gender for this sample was left unassigned. The sample tentatively identified as from a dog yielded no information on gender. Eleven swabs (61%) yielded ≥ 1 allele at one microsatellite locus. Three swabs yielded unambiguous, heterozygous microsatellite genotypes, and 1 swab was homozygous at both loci. Five swabs yielded heterozygous genotypes, and 2 swabs were homozygous at FH2137. Finally, 6 swabs yielded heterozygous genotypes, and 4 swabs were homozygous at FH2159. Using the criterion of only scoring alleles detected at least twice, none of the swabs yielded more than 2 alleles at a locus. Insufficient sample remained to replicate FH2010 genotypes, so we did not report them.

Discussion

We demonstrated methods for reliable species, gender, and individual genotype analysis of mixed

coyote: sheep samples and used those methods on swabs from predation wounds. Deposited saliva can be used as a DNA source, but caution must be taken in handling and analyzing such samples. Precautions similar to those recommended for other non-invasive DNA samples (such as scat, hair, etc.) should be taken, including appropriate sample preservation, avoiding sample contamination in the field or laboratory, and minimizing genotyping errors (Taberlet and Luikart 1999). Although DNA in deposited saliva may be subject to less bacterial degradation than scat-derived DNA, low amounts of template DNA indicate that multiple amplifications are necessary to confirm microsatellite genotypes. Indeed, we detected false alleles (amplification artifacts, Taberlet and Luikart 1999) in several of the saliva microsatellite amplifications and led us to our criterion that in order to be scored, alleles be detected at least twice. Small volumes of template DNA limited our ability to use more rigorous standards (such as performing more amplifications and scoring alleles only detected in more amplifications). Thus, although species identification based on high copy mitochondrial DNA may be readily performed, generating microsatellite genotypes likely will be limited by small amounts of template available from swabs. Obtaining multiple swabs from each puncture may potentially alleviate this by increasing available template DNA. Although we performed multiple amplifications in the analysis of the swab DNA, only single DNA extracts could be isolated for each swab. This is in contrast to some studies using scat DNA, where scat samples are large enough to allow multiple DNA isolations per sample.

We collected swabs that yielded genetic information in diverse environmental conditions, ranging from cool and rainy (high of 8°C, >1 inch of rain within approximately 24 hours prior to sampling the carcass) to hot and dry (high of 37°C, environmental data not shown). So, although environmental conditions may impact success rates of analysis of saliva DNA on predation wounds, it was likely that discovery of carcasses, successful identification and swabbing of predation wounds (versus scavenging), number of predators involved, and stochastic deposition of predator cells or DNA on the carcass will have a greater impact on results. Other limitations may include identification of suitably informative loci that reliably amplify low quantities of template DNA and additional costs associated with multiple amplifications.

Swabs from multiple punctures from each carcass may enable identification of multiple predators. Multiple predators would be identified by detecting different genotypes from different swabs of the same carcass or, for single swabs, more than 2 alleles being detected repeatedly at some microsatellite loci, or a combination. We did not identify any instances of multiple predators with these data.

In some instances predator species identification alone may be valuable, such as in areas where multiple canid or other species predate livestock. For example, techniques described here could be useful in determining the veracity of reports of livestock killed by wolves (*Canis lupus*).

The methods detailed here could be extended to other predator-prey combinations and also would be useful for other forensic applications. In the process of this study, we identified species-specific ZFX- and ZFY-RFLPs that allow discrimination of coyote gender in some mixed-species samples. Amplification of the canid-specific Sry region was more robust to variation in coyote: sheep DNA ratios than ZFX/ZFY amplification, and even large excesses of sheep DNA did not interfere with Sry analysis. Hence Sry was more useful with unknown mixed DNA samples, such as swabs from predation wounds. These methods should assist investigations into whether sex biases are apparent in predation behavior. For example, the majority (17/18) of our swab samples were from sheep killed by male coyotes. Given the conserved nature of the ZFX/ZFY and Sry regions, similar sexing markers should be useful in predation events involving other canid and prey species.

We are comparing microsatellite genotypes from the predation wounds with tissue genotypes from the coyote population in the area (Williams et al. 2003) and movement patterns and home-range information for individual coyotes to determine which coyotes were responsible for sheep predation at the study site, and their social and reproductive status. We expect that even the partial genotypes will be of use, in combination with that other information. Identification of the specific animals predating sheep will increase our understanding of coyote predation on sheep in general and may enable development of better strategies to minimize losses due to coyotes at this site. Individual predator identification through microsatellite genotyping of wound swabs also may be extremely useful in areas where reintroduced canids are being monitored.

This work demonstrates that traces of DNA remaining on wounds can be useful for confirming the species, gender, and even microsatellite genotype of the predator. Genetic analyses of predation wounds may provide researchers, wildlife managers, and producers with a powerful tool to better understand predation and manage predators.

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