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Preliminary assessment of DNA extraction methods and utility of microsatellite genetic assay for Southern California American badgers

By Dustin A. Wood, Anna Mitelberg, Cheryl S. Brehme, and Amy G. Vandergast

San Diego Field Station
USGS Western Ecological Research Center
4165 Spruance Road, Suite 200
San Diego, CA 92101

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Goals:

The goals of this study were to (1) conduct a preliminary assessment of alternative DNA extraction protocols to provide efficient isolation of DNA from badger scat samples, and (2) to assess the utility of a microsatellite genetic assay using available published microsatellite loci for identifying individual badgers in San Diego County.

DNA Extraction Tests

Sample collection

We used the scat and hair samples that were collected during the rapid assessment studies in 2011 and 2014 (Brehme et al. 2012, 2015) to evaluate several extraction protocols. For comparative purposes, we also obtained fresh badger scat from a captive badger at The Living Desert, Palm Desert, California, to assess whether the field collected scat samples yielded more degraded DNA than the fresh samples. Finally, we obtained liver and muscle from two specimens collected from road mortalities for use as positive controls.

Extraction Protocols

We evaluated two DNA extraction protocols to assess whether one method was more advantageous over the other for obtaining DNA from scat. The two kits used were the DNA IQ Kit (Promega) using the Tissue and Hair extraction protocol (hereafter DNA IQ kit) and the PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc).

For the DNA IQ kit, we evaluated three different modifications to remove epithelial cells off the outer surface of the scat: (1) PBS wash, (2) swab, and (3) vortex. For the PBS wash modification, we placed a 30 mm^3 piece of scat into a plastic weighing boat and gently washed the scat with 1xPBS. Because some PBS was absorbed by the scat, fresh PBS was added to the weighing boat as needed, with the goal of allowing as much of the surface of the scat (presumed to contain intestinal epithelial cells) to wash off and become suspended in the PBS solution, without allowing the scat to disintegrate. This washing process was continued for ~1-3 minutes and the PBS and intestinal cell solution was drawn up with a pipette and collected in a 1.7 ml centrifuge tube. For the swab modification, we simply swabbed the entire surface of the scat to remove the epithelial cells from the surface of the scat. For the vortex modification, we weighed out between 500 – 1000mg of scat in a 5ml tube, added 1xPBS until the material was covered, vortexed the sample for 1 minute, and then followed the standard DNA IQ protocol.

For the PowerFecal DNA kit, we weighed out approximately 300 mg of scat and followed the standard protocol. We also extracted DNA from muscle and liver, and hair samples obtained from several road-killed badgers and hair snag surveys from Southern California using the Qiagen DNEasy Blood and Tissue Kits (Qiagen, Inc), and used these DNAs as positive controls during sample assessments.

For each sample, we quantified the concentration of DNA using Qubit Fluorometric quantitation to compare the effectiveness of each extraction method.

Results

We obtained the highest concentrated DNA from scats with the PowerFecal Kit (Table 1). However, the higher DNA concentration obtained with this kit may result from the non-discriminate extraction of tissue, because both the outer and inner portions of the scat is used, rather than just the outer section as in the swab and PBS wash methods. Therefore, the total badger DNA from the PowerFecal kit may still be low relative to the total DNA recovered, as some of the recovered DNA may be from the diet of the badger. All other methods did not improve the DNA yield from the field scats. We did not detect much difference in DNA concentration between the various DNA IQ methods (Swab, PBS wash, and vortex) and none of the methods seemed to consistently improve the DNA yield. Similarly, there was no detected difference between fresh scats obtained from the captive badger and the field collected scat samples. We would recommend further exploration of available kits and protocols to better refine the potential of using scat as a source for badger DNA (Qiagen Stool Kit, Forensic protocols, ethanol precipitation, etc.), but the PowerFecal kit seems like the most promising method for acquiring DNA from badgers in San Diego County.

Although only one hair was collected during the rapid assessment study in 2014, this single hair (SDR23) was sufficient to yield an ample amount of high quality DNA to use with the microsatellite genetic assay (see below). We also confirmed the use of hair with one of the road mortality samples (BD4514).

Microsatellite Loci tests

The successful development of a badger specific microsatellite scat assay would enable us to learn much more about badger abundance and ranges of individual American badgers within the County. In 2012, the Center for Conservation Biology (CCB) at the University of Washington developed a badger specific assay to identify scat to the species level. They also conducted preliminary investigations to develop a microsatellite assay to identify individuals but were unable to amplify microsatellite loci from field collected scat DNA (Samuel Wasser, Rebecca Booth, pers. Comm). Prior to our testing, we consulted with CCB to review their initial work and recommendations.

For the microsatellite assays, we screened a total of 62 individual DNA samples. We used a total of 15 DNA samples from our extraction assays that yielded sufficient concentrations (>0.3 ng/ul) and the control samples we generated (see DNA Extraction tests above). In addition, we screened the 47 DNA samples that were obtained from CCB. These DNAs were extracted from field-collected scats from the 2011 and 2014 surveys and confirmed to contain American badger DNA using a badger specific mitochondrial DNA assay (Brehme et al. 2012, 2015).

Using these DNAs, we screened nine microsatellite loci obtained from the literature for use in developing a genetic assay for identifying individual badgers in San Diego County. These loci were originally developed in American badger (*Tt-1, Tt-2, Tt-3, Tt-4*; Davis and Strobeck 1998), American marten (*Ma-1*; Davis and Strobeck 1998), and European badger (*Mel1, Mel14, Mel101, Mel111*; Carpenter et al. 2003; Domingo-Roura et al. 2003). We selected these loci because of the demonstrated utility across species

(see references above) and the potential likelihood of working with low quality/degraded DNA given the relatively small size (i.e. < 200 base pairs) of the PCR product obtained from these loci.

We divided these loci into four groups. Within each group, 1-3 loci were simultaneously amplified (annealing temperature evaluated at 56°, 58, and 60° Celsius) with a Qiagen multiplex plus PCR kit and following recommended PCR conditions: 10 μL reactions contained 5 μL of Qiagen multiplex PCR Master Mix, 1 μL primer mix (containing 2 μM of each primer), 1 μL Q-solution and 2 μL of RNase-free water. Amplified products were genotyped at BATJ, Inc. (San Diego, CA) and Eton Bioscience (San Diego, CA) on an Applied Biosystems 3130 Genetic Analyzer using the LIZ 500 size standard. We used gene-marker v1.90 (SoftGenetics) to edit the raw allelic data and score allele sizes. We also ran "negative controls" (sample only containing water instead of DNA) with each PCR and genotyping run to identify and eliminate errors that are common in microsatellite assays via contamination or sample degradation.

Results:

A total of 61 samples were screened with the nine microsatellite loci. However, we were only able to consistently amplify and generate genotypes from the DNAs that came from muscle, liver, and hair tissues (Table 2). Although we recovered genotypes from several loci using the DNA obtained from individual scat samples, we were rarely able to recover genotypes from more than four loci per sample, with the exception of samples BD3S and BD57 (Table 2;) where up to six loci were genotyped. In general, scat samples showed chromatogram peaks for more than four loci, but the signal strength for these additional loci were much weaker than other scored loci. Therefore confidence in allele determination for these instances were considered low and not scored. We attempted several modifications to the PCR conditions, such as decreasing the annealing temperature, increasing the number of cycles, and increasing the number of microliters added to the PCR mix, but none of these alterations affected the results of the genotyping.

Recommendations and concluding remarks:

Aside from the PowerFecal extraction kit, our results suggest that most of the extraction methods we employed for isolation of DNA from badger scat resulted in DNA concentrations that were likely too low for genotyping analyses and potentially suffer from the presence of PCR inhibitors. However, we suspect that with further exploration of DNA extraction methods (in particular the 'Swab method') and a focused attempt at removing common PCR inhibitors found in stool samples (e.g., complex polysaccharides, bile salts, etc.), we could improve on this work and provide working concentrations of DNA for genetic identification of Southern California badger populations. We are currently testing new methods of DNA scat extractions using the Desert Tortoise and these tests may provide new protocols and refinements for testing on American badgers. However, our results also suggested that hair is a very usable source of DNA. Therefore, we recommend continued use of hair snags as a non-destructive genetic sampling method for the American badger. Although detections of badger occupied

burrows have been very rare in comparison to that of badger scat, our test showed that a single hair yielded as much or more DNA than scats and we were able to genotype the DNA from the hair for all nine microsatellite loci.

The results from the microsatellite assay suggested that the PowerFecal extraction protocol appears to be the most consistent in recovering usable genotypes; we were able to recover between four and six genotypes from the two samples we extracted with this protocol (Table 2). We also would recommend further work to assess the 'Swab method' using the DNA IQ kit, as a least one sample yielded six amplified loci. For most other extractions we recovered weaker chromatogram signals that may be related to the DNA quality and quantity that was obtained from scat material. The fact that different loci were amplified across different scat DNAs (a phenomenon know as 'allele dropout') suggests that the specificity of the primer for each locus may be an issue. That is, the sequence match between the PCR primers and Southern California badger DNA may be low for some loci, which can cause inconsistent primer attachment and amplification of the desired DNA sample, resulting in the 'allele dropout' we observed across samples. This inconsistency can be especially problematic with samples of low DNA concentration. One method that has been commonly employed to circumvent this issue has been to perform multiple amplifications (at least 5 times) of the entire set of microsatellites and use a variety of computational methods to identify and eliminate errors.

A preferred potential solution for increasing the feasibility of genotyping low concentration/ low quality DNA with microsatellites would be to design a species-specific microsatellite library for Southern California badgers. As we have noted already, the microsatellite loci we tested were originally developed for several different species (American marten and European badger) and a different subspecies of the American badger from Alberta, Canada, which may also contribute to the allele dropout we observed. Developing a microsatellite library from tissues taken from Southern California would increase the primer specificity for the local badger populations and potentially increase our ability to use DNA from degraded sources (e.g., well preserved scats). The USGS Genetics facility at the San Diego Field Station is uniquely poised to have the instrumentation necessary to develop a microsatellite enriched library specifically for Southern California American badger populations. Using our 454Jr-automated DNA sequencer (Roche), we can develop a microsatellite library for the local badger populations that should contain at least 100 microsatellites. From this library, we could select 10-20 polymorphic loci for further genotype development on the local populations of badgers. In addition, given that we are recommending the use of the PowerFecal extraction kit and that this extraction kit does not discriminate between badger DNA found *on* the scat and DNA present from the American badgers diet that may be *embedded in* the scat, we would also recommend screening the microsatellite loci against local species of prey that may be found in the American badger's diet. This would eliminate any loci that may amplify local mammal DNA present in badger scat.

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Table 1. Representative tissue samples, recovered DNA concentrations, and DNA extraction method evaluated for this study.

DNA Sample	Tissue Type	Concentration (ng/ul)	Extraction Kit	Method
SD3793	muscle	>60	Qiagen	Blood & Tissue Kit
SD3958	muscle	>60	Qiagen	Blood & Tissue Kit
SDR23	hair	15.0	Qiagen	Blood & Tissue Kit
BD4514	hair	0.47	Qiagen	Blood & Tissue Kit
BD4514	liver	>60	Qiagen	Blood & Tissue Kit
BD33015	liver	19.20	Qiagen	Blood & Tissue Kit
B1S	scat	0.13	DNA IQ	Swab
B2S	scat	0.05	DNA IQ	Swab
B3S	scat	0.66	DNA IQ	Swab
B1	scat	0.09	DNA IQ	PBS Wash
B2	scat	0.37	DNA IQ	PBS Wash
B3	scat	3.78	DNA IQ	PBS Wash
BD1	scat	5.22	DNA IQ	Vortex
SDR 24	scat	0.94	DNA IQ	Vortex
SDR01	scat	0.97	DNA IQ	Vortex
BD414	scat	12.30	MoBio	PowerFecal
BD57	scat	55.00	MoBio	PowerFecal

Table 2. Genotypes for nine microsatellite loci screened from DNA obtained from control samples and badger scats using various DNA extraction methods. Each microsatellite locus is listed by the published name (e.g. Tt-1, Tt-2, etc.) and the sizes of the two alleles are given under the columns A and B for each locus. The last column summarizes the number of loci genotyped for each DNA sample.

		Microsatellite Loci																		
		Tt-1		**Tt-2**		**Tt-3**		**Tt-4**		**MEL1**		**MEL14**		**MEL111**		**MEL101**		**Ma-1**		
DNA Sample	**DNA Source**	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	Number of Loci genotyped
SD3793	Muscle	166	166	203	203	153	165	168	180	262	272	179	191	140	146	133	139	196	196	9
SD3958	Muscle	160	166	203	203	153	153	180	180	262	262	183	191	146	146	--	--	--	--	7
SDR23	Hair	156	156	203	203	163	165	186	190	258	268	181	181	138	140	135	141	196	196	9
BD4514	Hair	160	162	203	203	153	165	180	186	--	--	181	183	146	146	139	143	196	200	8
BD4514	Liver	160	162	203	203	153	165	180	186	--	--	181	183	146	146	139	143	196	200	8
BD33015	Liver	166	168	203	203	153	167	190	190	264	264	177	179	144	148	137	139	200	202	9
BD168D	Scat (Swab)	158	158	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1
BD175D	Scat (Swab)	--	--	--	--	153	153	--	--	--	--	--	--	--	--	139	139	200	202	3
B3S	Scat (Swab)	154	154	--	--	163	163	168	180	262	272	179	179	--	--	--	--	196	196	6
B1	Scat (Wash)			--	--	--	--	--	--	--	--	183	183	--	--	--	--	200	200	2
B2	Scat (Wash)	--	--	--	--	--	--	168	180	--	--	--	--	--	--	--	--	--	--	1
SDR24	Scat (Wash)	--	--	--	--	--	--	--	--	262	272	181	191	--	--	--	--			2
BD414	Scat (PowerFecal)	--	--	203	203	--	--	168	168	--	--	179	179	--	--	137	141	--	--	4
BD57	Scat (PowerFecal)	154	154	203	203	--	--	168	168	262	262	177	177	--	--	--	--	196	196	6
BD1	Scat (vortex)	154	154	--	--	--	--	--	--	--	--	--	--	146	146	141	145	--	--	3
SDR24	Scat (vortex)	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0
SDR01	Scat (vortex)	--	--	--	--	--	--	--	--	--	--	--	--	150	150	--	--	--	--	1