Evaluation of Mandrill Monkey Fecal Preservation and Extraction Methodologies Reveal Ideal Combinations for Maximum DNA Yields

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ABSTRACT

Genotyping analysis using DNA obtained through non-invasively collected fecal samples can provide valuable information on most wildlife species. However, genotyping errors including allelic drop-out are common when fecal samples yield lower quantity and quality DNA. Several studies have produced conflicting or inconsistent results across fecal DNA preservation and extraction methodologies. In order to establish a simple, convenient, and consistent methodology that produces high DNA yields, preservation and DNA extraction techniques were compared across fecal samples obtained from mandrills (*Mandrillus sphinx*) at the Buffalo Zoological Gardens. All preservation methods provided adequate DNA extraction yields, though we strongly suggest the use of RNAlater in combination with QIAGEN extractions based on several considerations.

Fecal sampling has become a well-established tool for conservation genetic studies of primate populations and most other taxa because of advantages associated with non-invasive methods and relative ease of sample collection ( ) even though fecal samples may provide low or degraded template DNA for genotyping studies. The disadvantages include poor template DNA resulting in low or incomplete amplification (Smith et al., 2000), allelic drop-out, and reduced detection of heterozygosity (Lawler et al., 2001). However, increased DNA yield can offset many of these problems resulting in decreased allelic dropout rates and increased positive PCR amplifications (Morin et al. 2001). Several studies have examined fecal preservation methods to address the problem of sample degradation and low DNA yields with varying results, with considerable disagreement existing over which preservation and extraction methods provide the best results.

The present study investigates which combination of fecal sample preservation (air-dry, RNAlater, silica gel beads or 'two-step') and DNA extraction methods (QIAGEN and Dyanbeads) yield the most DNA in a carefully controlled setting. Fecal samples were obtained between February and August 2005 from one male and four female mandrills housed at the Buffalo Zoological Gardens in Buffalo, New York. A total of 45 fecal samples were collected and each sample was split into four subsamples. Each subsample was placed into a 50 ml tube and subjected to one of four preservation conditions (dry, RNAlater®, silica gel beads or "two-step") at ambient temperature (average temperature 23°C) for 3-4 weeks. Subsamples were arbitrarily assigned preservation type to avoid bias. For the silica gel bead method 20 gm of silica gel beads (Sigma-Aldrich) were placed in the bottom of the tube and covered with a kimwipe. The two-step method consisted of an ethanol soak (30 ml of 97% ETOH) for 24-36 hours, which was poured off and replaced with 20 g of silica gel beads as in the silica gel bead method. Samples subjected to RNAlater® (Ambion) preservation were added to 10 ml of RNAlater liquid and remained submerged throughout the 3-4 week storage period. RNAlater was poured off samples prior to freezing. All preserved samples were then stored for up to three months at -80°C after the ambient temperature preservation period.

Preserved subsamples were thawed on ice and then subjected to either a QIAGEN QIAamp DNA Stool Mini Kit extraction (cat. no. 51504) or a Dynabeads DNA Direct Universal extraction (cat. no. 630.06). For each pair of extractions 200 mg of the outer epithelial layer was scraped into two weigh boats, alternating approximately every 50 mg to equalize material. Samples were then randomly assigned to either QIAGEN or Dynabeads

extraction. All preservation conditions for a single initial fecal sample were extracted with both methods on the same day.

All DNA extractions were quantified with the VersaFluor™ Fluorometer System (BIORAD) according to manufacturer's protocol. To limit confounding variability in fluorometer measurements, all extractions for any one sample were assayed together in sets of 8, representing 4 preservation x 2 extraction methods. The fluorometer measures all DNA in the fecal sample, including unintended bacteria and potential food items. Therefore, a portion of the mitochondrial cytochrome *b* gene was sequenced to verify origins of the DNA. The universal primers mcb398 and mcb869 identified by Verma and Singh (2003) were used to amplify a 472 bp segment of cytochrome *b* that is highly specific to species-level animal identification. A random subset of seven samples from each extraction type were used as template DNA for a 50 µl reaction volume containing approximately 50 ng of template DNA, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 25 pmol of each primer, 2.5 U *Taq* DNA Polymerase (New England Biolabs), and 1x PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl). The PCR conditions were: 95°C for 10 min, followed by 35 cycles of 95°C for 45 sec, 51°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 8 min and a 4°C hold. Amplified fragments were sequenced in both directions with the Quick Start DTCS kit (Beckman Coulter) and the nucleotide bases were separated on a Beckman Coulter CEQ8000 Genetic Analysis System. The resulting sequences were compared against *nr* databases of National Centre for Biotechnology Information (NCBI) using BLAST (Altschul et al., 1997).

Following the nomenclature of Milliken and Johnson (1984, p.76), the total DNA yield was analyzed as a split-plot, in which the whole plot experimental units were a

randomized complete block design. Each of the 45 fecal samples was treated as a "replication" containing four homogeneous blocks. Each block within a replication was randomly assigned to one of the four preservation conditions and was considered a "whole plot." Because each block was then split and subjected to one of the two extraction methods, the two parts of each block were considered "subplots" within the whole plot block. In the overall design, fecal samples (i.e. replications) were treated as random factor and preservation conditions (i.e. whole plots) and extraction methods (i.e. subplots) were treated as fixed factors. The sample*preservation interaction served as the error term for the main effect of preservation (i.e. the whole plot), and the sample*preservation*extraction interaction served as the error term for the main effect of extraction (i.e. subplot) and the preservation*extraction interaction. The data were analyzed with the General Linear Model's univariate procedure in SPSS (version 14.0 Release 14.0.2, March 2006). Data were first square root transformed ($Y' = \sqrt{Y+1}$) to meet the assumptions of normality and equality of variances.

DNA concentration varied from 0 ng/µl to 147.9 ng/µl. The average DNA concentration across all samples was 15.5 ng/µl. There was no effect of preservation method on DNA yield ($F_{3,132} = 1.874$, P=0.137), and the interaction between preservation and extraction methods also was not significant ($F_{3,176} = 0.745$, P=0.527; Fig. 4) . Tests of the main effect showed that extraction type for each preservation method was highly significant, underscoring the enhanced yield for QIAGEN extracted samples ($F_{1,176} = 61.91$, P=0.0001 for air-dry ; $F_{1,176} = 87.57$, P=0.0001 for RNAlater; $F_{1,176} = 61.06$, P=0.0001 for silica gel beads; $F_{1,176} = 86.72$, P=0.0001 for two-step).

QIAGEN samples had the highest DNA concentration (21.8 ng/µl) and total DNA yield (4.3 µg/extraction) on average (Table 1). Only six (3.3%) QIAGEN-extracted samples yielded no measurable DNA, compared to 19 (10.6%) Dynabead-extracted samples. Seventy-one percent of QIAGEN-extracted samples provided DNA concentrations > 5 ng/µl, while only 21% of Dynabead-extracted samples had DNA concentrations > 5 ng/µl. The extraction method had a significant effect on DNA yield ($F_{1,176} = 295.028$, $P < 0.001$; Table 2); samples extracted with QIAGEN yielded approximately 11.5 times more DNA than did samples extracted with Dynabeads regardless of preservation method (mean = 4351 ng and 377 ng, respectively; Fig. 4).

None of the seven Dynabeads-extracted DNA samples yielded amplified product from the initial cytochrome *b* PCR reaction. All seven of the QIAGEN-extracted DNA samples yielded PCR fragments and four of these resulted in complete sequence of the 472 bp region of cytochrome *b*. These four sequences were submitted to the NCBI database (Accession numbers EU939973, EU939974, EU939975, and EU939976). Incidentally, two single nucleotide polymorphisms were discovered that provided individual animal identification.

By using mandrill samples from a controlled zoo setting, we were able to attribute differences in DNA yields to preservation and extraction methods rather than diet, weather and collection practice. Our findings suggest the QIAGEN QIAamp mini stool kit is a far better fecal DNA extraction method than Dynabeads DNA Direct Universal. Not only did the QIAGEN extraction yield more DNA in a larger volume, it also produced better quality DNA, as evidenced by the proportion of successful primate sequences generated from a

random set of sub-samples versus no amplified cytochrome *b* product from Dynabeads samples.

Although there were no significant differences between the DNA yields based on preservation method, there is variation in the number of extractions available. Air dry, silica gel beads, and 'two-step' preservation methods desiccate the sample, which shrinks it and makes it friable. RNAlater is an aqueous solution and the fecal pellet maintains its shape and size. In essence, relatively little sample was used in RNAlater conditions to obtain comparatively similar DNA yields to desiccated samples. Our results showed no significant difference in average DNA concentrations between silica (17.9 ng/uL) and RNAlater (25.6 ng/uL) when using Qiagen extraction kits. QIAGEN QIAamp DNA Stool Mini Kit performed significantly better than Dynabeads DNA Direct Universal extractions, by a 12:1 margin. That difference is even more significant since QIAGEN extractions are eluted in 200 µl, versus 40 µl for Dynabead extractions.

We expect this work from a zoo setting will prove insightful for field collections, particularly those aimed at primate populations, even though differences will undeniably exist between captive and wild settings.

Research protocols were approved by the Buffalo Zoo RCC/IACUC and adhered to the legal requirements for non-human primate research in the USA.

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229

TABLES

Table 1: DNA concentration in ng/µL for Qiagen and Dynabeads extractions for each preservation method.

DNA Concentration	Qiagen (ng/µL)	SD	Dynabeads (ng/µL)	SD
Dry	17.9	20.4	7.0	11.0
RNAlater	25.6	30.1	9.0	14.3
Silica gel beads	20.5	24.5	12.2	20.7
2-step	23.1	21.8	9.5	13.7
Mean	21.8	24.5	9.4	15.3

Table 2: Analysis of Variance on DNA yields in fecal samples. Analysis was carried out on square root transformed total yield using a split-plot design, in which there were $s = 45$ samples, $a = 4$ preservation methods (whole plot), and $b = 2$ extraction techniques (subplot).

Source of Variation	*df*		*SS*	*MS*	*F*	*P*
Whole Plot Analysis						
Sample	$(s\text{-}1)$	44	85573.042			
Preservation	$(a\text{-}1)$	3	1993.520	664.507	1.874	0.137
Sample * Preservation	$(s\text{-}1)(a\text{-}1)$	132	46804.771	354.582		
Subplot Analysis						
Extraction	$(b\text{-}1)$	1	162934.339	162934.339	295.028	0.000
Preservation * Extraction	$(a\text{-}1)(b\text{-}1)$	3	1233.840	411.280	0.745	0.527
Error	$a(s\text{-}1)(b\text{-}1)$	176	97199.203	552.268		

FIGURES

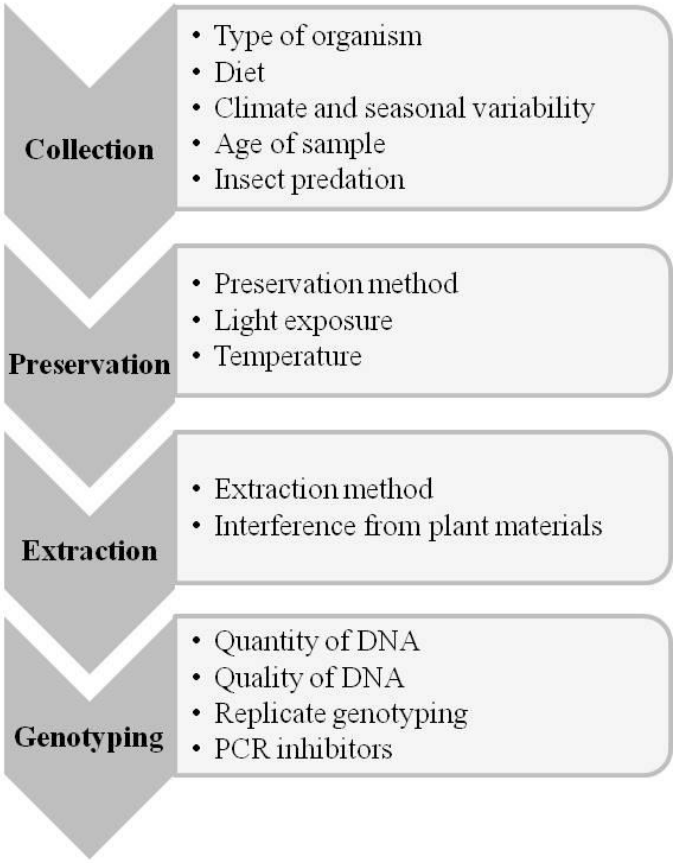

Figure 1: Fecal Genotyping success is dependent on collection, preservation, and extraction techniques. These factors each have the ability to influence the quantity and quality of DNA, which is instrumental in increasing genotyping success.

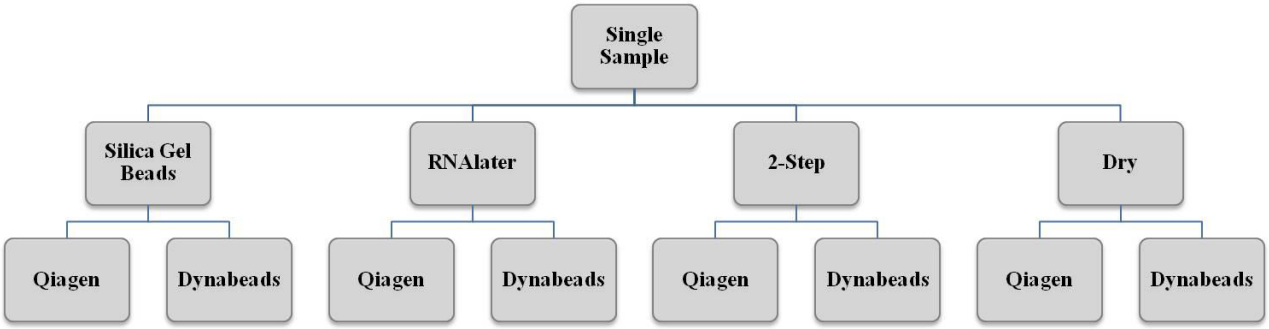

Figure 2: Experimental preservation and extraction design. Mandrill fecal samples were collected at the Buffalo Zoological Gardens (top row) and split four ways (middle row). When the preserved samples were extracted, the outer layer was scraped equally into two weigh boats. Each sub-sample was then arbitrarily assigned to either Qiagen QIAamp DNA Stool Mini Kit or Dynabeads DNA DIRECT Universal extractions (third row).