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Viability of fecal DNA extracted from spotted deer's fecal samples preserved in technical grade ethanol for non-invasive DNA analysis

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ABSTRACT. Fecal sample is now commonly used in molecular studies. Fecal sample is commonly used in wildlife molecular studies as it is a non-invasive method. Although fecal DNA have some shortcoming, several optimizations have been made for molecular analysis using fecal DNA. In this study, we used fecal samples preserved in technical grade ethanol, which were a suboptimal condition. This study aims to determine whether a suboptimal preservation is viable for DNA extraction and PCR amplification and which method is more effective. We used QIAamp Fast DNA Stool Mini Kit (QIAGEN) and compared three different modification of extraction method. DNA quantity was measured using Tecan Spark NanoQuant Plate™. Following that, we amplified the DNA with PCR procedure using three different primer sets. DNA was amplified successfully with a good quality result for primers that target smaller size of sequence (around 500bp). Extraction using surface scrapping method yielded better average DNA quality compared to the other two method in both DNA concentration (17.54ng/µL compared to 5.99ng/µL and 6.33ng/µL) and purity (2.84 compared to 36.21 and 9.22). DNA purity showed to be the main factor affecting amplification success among the parameters used in this research. The surface scrapping method successfully yielded DNA from the fecal samples preserved in technical grade ethanol that were viable for PCR amplification and sequencing.

Keywords: DNA extraction method; fecal DNA; molecular analysis; PCR amplification; spotted deer

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INTRODUCTION

Fecal sample is now commonly used in molecular studies, especially for wildlife studies. Fecal sample is often used because it's relatively easy to collect, as it is a non-invasive method. Some extraction kit has been developed which specialized in DNA extraction from fecal sample. Fecal DNA has become an increasingly used source of information to study population (Méndez *et al*., 2014; Laguardia *et al*., 2015; Ruiz-Gonzalez *et al*., 2015; Gil-Sánchez *et al*., 2017; Norman *et al*., 2017), species identification (Joo & Park, 2012; Woodruff *et al*., 2014; Walker *et al*. 2016; Verkuil *et al*., 2018; Walker *et al*., 2019), gut microbiomes (Chi *et al*., 2019; Gibson *et al*., 2019; Oliveira *et al*., 2020) and also help behavioral study (Bischof *et al*., 2016; Forcina *et al*., 2019).

Although fecal DNA is used more often nowadays, fecal DNA have some shortcoming. A common occurrence in genetic analysis using fecal DNA is the Polymerase Chain Reaction (PCR) inhibition. Stool may contain a lot of inhibitors that can obstruct PCR process, such as polysaccharides, bile salts, lipids, and urate, which could lead to an amplification failure (Schrader *et al*., 2012; Angelakis *et al*., 2016; Acharya *et al*., 2017). A comparison of short tandem repeat loci extracted from blood and fecal samples of American bison showed a significant decrease in heterozygosity estimates from the fecal samples (Forgacs *et al*., 2019).

Several comparisons have been done for the optimization of molecular analysis using fecal DNA. A modification on the preservation of sample shows varying results. Several successful preservation methods include freezing, storage in molecular grade ethanol, FTA card, buffers such as EDTA and lysis buffer, and also DNA/RNA Shield™ (Bahl *et al*., 2012; Carozzi & Sani, 2013; Song *et al*., 2016; Menke *et al*., 2017; Kazantseva *et al*., 2021; Bach *et al*., 2022). An optimization on the sample collection method had been published and compared, which are the collection of whole pellet or fragment and surface swabbing (Ramón-Laca *et al*., 2015; Sarabia *et al*., 2020). The use of different

extraction kit also showed to have significant effect on the concentration, purity, and performance on PCR, especially for microbiome analysis (Leite *et al*., 2013; Fock-Chow-Tho *et al*., 2017; Lim *et al*., 2020).

Although several optimizations had been done which had been proven successful, several reagents or kit might not be available for some researcher. An impromptu sample collection by field practitioner and citizen might not have the proper materials readily available. This suboptimal condition might make further molecular analysis difficult. In this research, we extracted the DNA from spotted deer's fecal samples preserved in technical grade ethanol, which are commonly available to be purchased from suppliers in Indonesia. Technical grade ethanol is ethanol with relatively high impurities (Tse *et al*., 2021). Technical grade ethanol might contain several molecules mixed into the ethanol which might inhibit further process. Previous studies have described the optimal procedures for molecular analysis using fecal DNA. This research shows a viable method for using fecal samples preserved in technical grade ethanol, which is a suboptimal condition, to be used for further molecular analysis. We compared several modifications of DNA extraction method using the QIAamp Fast DNA Stool Mini Kit (QIAGEN). This study aims to determine whether a suboptimal preservation is viable for DNA extraction and PCR amplification and which method is more effective.

MATERIALS AND METHODS

Sample. Spotted deer's fecal samples used in this research were obtained from another research project. The samples were collected from Gembira Loka Zoo (7.8077° S, 110.3958° E), PIAT UGM (7.7961° S, 110.4653° E), Prambanan Temple (7.7520° S, 110.4915° E), and Bogor Palace (6.5980° S, 106.7975° E). Three to four whole fecal pellets were collected as samples and preserved in 20mL ethanol labeled as 96% technical grade ethanol inside a sterile 30mL stool container. The stool containers were then stored inside a box in room temperature until DNA extraction. Nineteen samples were used for this research. A fresh fecal sample from a pet cat was also collected as a comparison for the DNA extraction and amplification. A DNA extracted from bat tissue was also used as a comparison in PCR amplification.

DNA extraction. DNA extraction was done using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, cat. no. 51604) manufacturer's protocol with some modifications (Sarabia *et al*., 2020). We made three different modifications to the method to compare which method is more effective as follows. **Method 1.** Three whole fecal pellets were homogenized with the ethanol. The rest of the procedure was done according to the QIAamp Fast DNA Stool Mini Kit (QIAGEN) protocol, with modification of using 100µL ATE buffer instead of 200µL. **Method 2.** Three whole pellets were homogenized by vortexing and 800µL were pipetted into 2ml microcentrifuge tube. It was then centrifuged at 13,000 rpm for 1 minute and the following procedure was done according to the QIAamp Fast DNA Stool Mini Kit (QIAGEN) protocol with some modification. The starting materials used was 400µL of the supernatant. InhibitEX used was modified to 500µL and vortexed for 10 minutes. A spin down process was added, followed with 30 minutes waiting time. Proteinase-K used was modified to 30µL and vortexed at low speed instead. An incubation step was added at 70°C for 10 minutes after addition of proteinase-K. Vortex time after addition of AL buffer was modified to 1 minutes. Incubation period after the addition of AL buffer was modified to 1 hour, with mixing after for every 20 minutes. Vortex time after the addition of ethanol was modified to 10 minutes and followed by incubation in the freezer for 1 hour. Centrifugation when loading the lysate to the spin column was modified to 8,000 rpm. The rest of the centrifugation was done at 10,000 rpm. A resting period with open cap was added after washing steps for 30 minutes. ATE buffer was incubated at 56°C before elution, and modified to be 85µL. **Method 3.** For the third method, the preserved fecal samples were removed from the ethanol and the surface was scrapped using a sterile scalpel. The scrapped surface was then collected in total of about 160-240mg materials. The rest of the procedure was identical to the protocol from QIAamp Fast DNA Stool Mini Kit, with a

modification of using 100µL ATE buffer instead of 200µL at the end. The eluted DNA was then stored in a freezer.

DNA Quantification. For the DNA quantification, we send the eluted DNA to LPPT UGM to be analyzed. Quantification was analyzed using the Tecan Spark NanoQuant Plate™ for the DNA concentration (ng/µL) and purity at 260/280 nm absorbance (A260/280). **PCR Amplification.** For the amplification we used three set of primers, which are ML103 forward primer (5′-GACTAATGATATGAAAAACCATCGTTG-3′) and MH104 reverse primer (5'-TTGTTCTTCATCTCTGGT TTACAAGAC-3') (Chikuni *et al*., 1995) which is a universal mammal cytochrome b primer with product size of around 1140bp (Świsłocka *et al*., 2013), L15995 forward primer (5′-CTCCACTATCAGCACCCAAAG-3′) (Taberlet & Bouvet, 1994) and H16498 reverse primer (5′-CCT GAAGTAAGAACCAGATG-3′) (Fumagalli *et al*., 1996) which is a universal D-loop primers with product size of around 500bp (Harsini *et al*., 2017), and DLF (5′-AGCCTCACTATCAACACCCA-3′) and DLR (5′-CACATAGGTTTGGTCCCAGC-3′) (Abbas *et al*., 2016) which is a D-Loop primer with a product size of 1020bp designed specifically for spotted deer. The PCR reaction with a total amount of 25µL contained 12.5µL of MyTaq™ HS Red Mix (Bioline), 1 mM $MgCl_2$, 0.4 µM each of forward and reverse primer, 4.5µL ddH_2O, and 5µL DNA template. The phases of the DNA amplification PCR profile following Arisuryanti *et al*. (2020) include pre-denaturation of the template at 95°C for 1 minutes, denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, repeated for 35 cycles, and final extension at 72°C for 10 minutes.

PCR product visualization. PCR product was visualized using electrophoresis on 1% agarose gel using FloroSafe DNA stain (1st BASE). A total of 2µL PCR product was inserted into each well. The electrophoresis machine was run at 100 volt for 25 minutes and the results were visualized using a UV fluorescent (Kasayev & Arisuryanti, 2022).

Data analysis. We count the storage time of the samples by the number of days from sample collection to DNA extraction. A two-tailed bivariate correlation between DNA concentration, DNA purity, amplification success, and storage time was done with IBM SPSS ver. 25, using Spearman's correlation for two numerical variable and point-biserial correlation for the correlation between a numerical and a categorical variable.

RESULTS AND DISCUSSION

DNA extraction yields. DNA quantification result is shown on Table 1. Extraction using method 1 yields a mean concentration of 5.99 ng/µL and a mean purity level of 36.21. Extraction using method 2 yields a mean concentration of 6.33 ng/µL and a mean purity level of 9.22. Extraction using method 3 yields a mean concentration of 17.54 ng/µL and a mean purity level of 2.84. DNA extracted from deer fecal sample with method 3 a relatively close result with fecal DNA extracted from a fresh cat stool (Fig. 1).

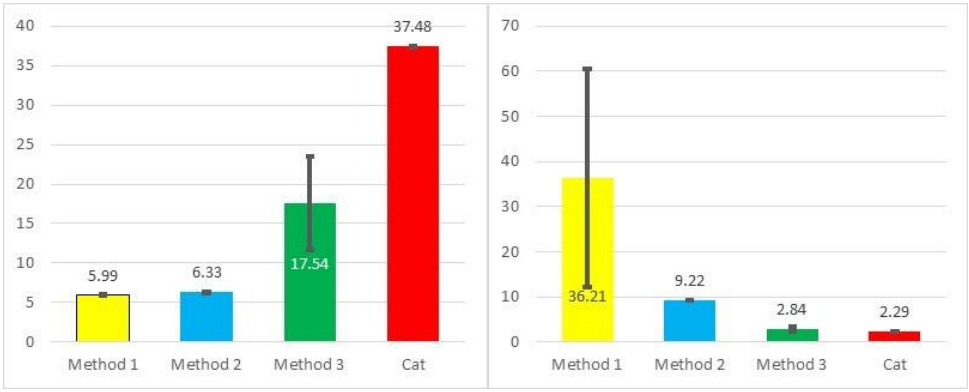

Fig. 1. Quantification of DNA yields: a. Concentration (ng/µL); b. Purity (A260/280).

Previous research shows DNA concentration seems to varied greatly among different kit used, different method, and also different species. Previous research with similar method and kit shows mean concentration of 7.776 (± 5.610) ng/µL for white-tailed deer and 31.903 (± 42.356) ng/µL for fallow deer (Ramón-Laca *et al*., 2015). The result of this research showed concentration values between those two species. Since DNA concentration varies significantly between factors and no publication had shown DNA concentration yields for spotted deer in similar methods, a direct comparison could not be done. In theory, a pure DNA should have a A260/280 value of ~1.8 (Lucena-Aguilar *et al*., 2016). In this study, method 3 yielded the closest purity to the ideal value, which makes method 3 better than the other method used in this research. Even so, amplification success is still the most important thing to consider as the purpose is for DNA analysis.

Table 1. DNA quantification results using Tecan Spark NanoQuant Plate™.

Sample	Concentration	Purity (260/280)
Deer A1	5.93	53.3
Deer A2	6.04	19.12
Deer B1	6.33	9.22
Deer C1	23.79	2.44
Deer C2	13.97	3.36
Deer C3	19.83	2.82
Deer C4	11.09	3.33
Deer C5	11.96	3.41
Deer C6	14.22	2.85
Deer C7	16.96	1.86
Deer C8	15.37	2.83
Deer C9	21.46	2.81
Deer C10	15.63	3.03
Deer C11	19.88	2.67
Deer C12	27.34	2.48
Deer C13	31.12	1.33
Deer C14	12.1	3.75
Deer C15	13.22	3.35
Deer C16	12.66	3.17
Cat	37.48	2.29

Note: A. Method 1; B. Method 2; C. Method 3

PCR amplification. All of the DNA extracted with method 1 and 2 failed to amplify. A comparison of PCR quality from sample extracted with method 3 was done with fecal DNA from fresh cat stool and DNA extracted from bat tissue obtained from another project (Fig. 2). Amplification using the L15995 and H16498 primers yielded a good band result on all three samples. Amplification using the ML103 and MH104 primers yielded a good result on the bat DNA but produced a thin band on the cat fecal DNA. It did not amplify successfully on the deer fecal DNA. Amplification using the DLF and DLR primers yielded a small size double band, which did not match the supposed target.

The amplification using bat DNA yielded better results, which was shown by the clearer band compared to the fecal samples result. This is caused by higher DNA concentration and better DNA purity of the DNA template, as it was extracted from tissue which was a better source of DNA than fecal samples. Several publications had addressed the shortcoming of using fecal DNA for analysis. A comparison of DNA extracted from blood and fecal samples in bison showed that the DNA extracted from fecal sample have a significantly higher allelic dropout rate (Forgacs *et al*., 2019). This could indicate a disadvantage of using fecal samples for DNA analysis in general. Even so, a comprehensive comparison of various sample source which included fecal sample had not been published.

From the fecal samples, amplification was successful for primers that target lower size of sequence, while those that target longer sequence failed to amplify or amplified poorly. This could be caused by several reasons, which could be insufficient DNA concentration, DNA damage and

fragmentation, or presence of PCR inhibitors. Further optimization of PCR program might successfully amplify the DNA, but which could not be proven as it was not tested in this research.

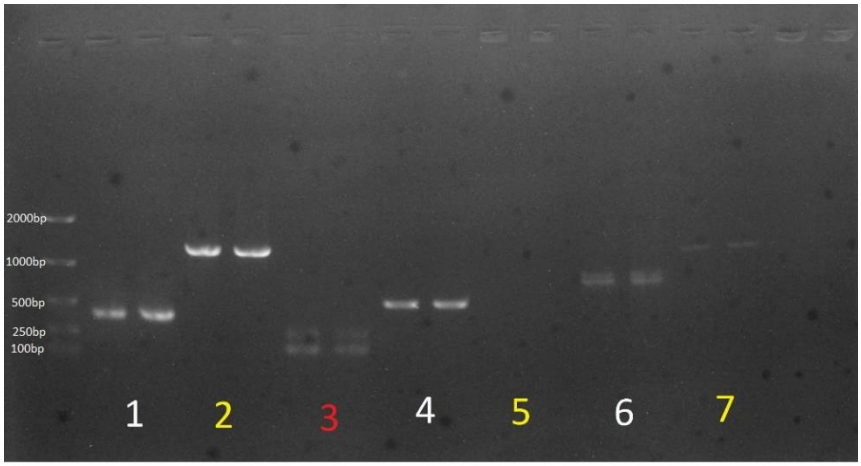

Fig. 2. Amplification results: 1. Bat DNA using L15995 & H16498 primers; 2. Bat DNA using ML103 & MH104 primers; 3. Deer fecal DNA using DLF&DLR primers; 4. Deer fecal DNA using L15995&H16498 primers; 5. Deer fecal DNA using ML103&MH104 primers; 6. Cat fecal DNA using L15995&H16498 primers; 7. Cat fecal DNA using ML103&MH104 primers.

The high content of polysaccharides in the diets of spotted deer constitute to the high concentration of PCR inhibitor in its feces. These polysaccharides are usually concentrated on the central part of the pellet. Surface scrapping of the pellet reduced the polysaccharides included in the DNA extraction process, which minimalized inhibitor co-purification and minimalized PCR inhibition. Previous publication had shown the effectiveness of surface scrapping method of fecal samples compared to using the whole pellet (Ramón-Laca *et al*., 2015). The unsuccessful amplification of DNA obtained from method 1 and 2 might also be caused by the impurity of preservative ethanol, as preservative concentration could affect DNA extraction and amplification success (Marquina *et al*., 2020). This might be avoided by using different preservative, such as lysis buffer.

Amplification using L15995 and H16498 primers was further done to other samples extracted with method 3. Out of 16 samples, 4 samples failed to amplify while 12 samples amplify successfully. Out of those 4 samples, 2 have a relatively low DNA purity compared to the other samples, which might be causing the amplification failure. The other 2 samples did not show a significant difference from the other samples which were successfully amplified. The amplification failure of these 2 samples might be caused by several other reasons, for example the presence of PCR inhibitors, which was not tested in this research. Further consideration regarding factors affecting amplification success is discussed on the next paragraph referring to Fig. 3.

There is no significant correlation between sample storage time and both DNA concentration and purity (0.973 and 0.725 significance on 2-tailed analysis respectively). This indicates that samples preserved in this technical grade ethanol are still viable for molecular analysis for at least up to 2.5 months stored in room temperature after collection. Storage time also shows no significant correlation with amplification success (0.850 significance on 2-tailed analysis). DNA concentration shows no significant correlation with amplification success (0.057 significance on 2-tailed analysis). Even if the total DNA concentration is high, some of that total DNA might be from a different target, including DNA from different creatures, hence it does not correlate directly with amplification success. DNA purity and amplification success shows a significant correlation at the 0.05 level (2-tailed) with a significance of 0.010. From the scatter plot, purity level of 2.4-3.5 could amplified successfully, while those with lower purity level failed to amplify. Impurity of DNA yield could suggest contamination, which could be a PCR inhibitor.

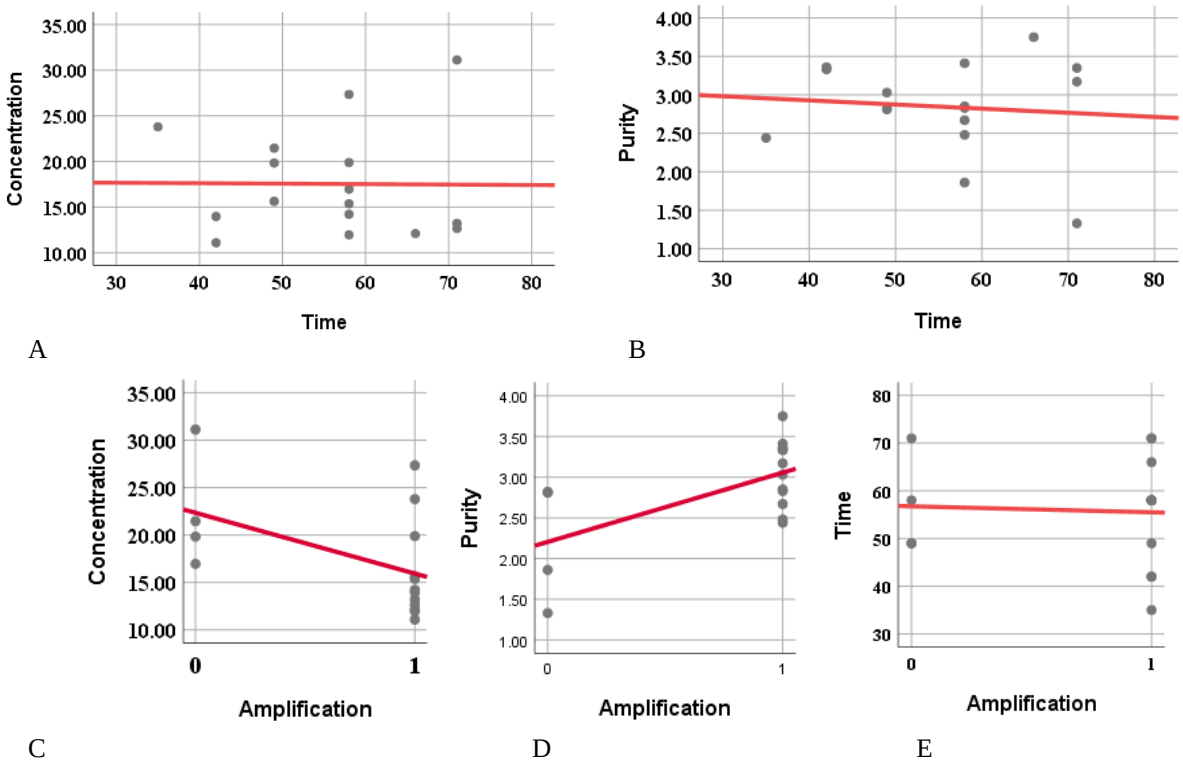

Fig. 3. Bivariate correlation between variables: a. DNA concentration (ng/µL) & sample storage time (days); b. DNA purity (A260/280) & sample storage time (days); c. DNA concentration (ng/µL) & Amplification success (0: failed; 1: succeed); d. DNA purity (A260/280) & Amplification success (0: failed; 1: succeed); e. Sample storage time (days) & Amplification success (0: failed; 1: succeed).

Previous studies have observed a different performance of sample collection and DNA extraction method based on the diets of the animal. For herbivore which consumes a high amount of polysaccharides in their diet, surface swabbing and preservation in Longmire's lysis buffer might be better, while for carnivore, processing the whole fecal fragment yields a better result (Ramón-Laca *et al*., 2015; Sarabia *et al*., 2020). Even though not in an optimal condition, all successfully amplified samples from this research could be sequenced successfully and showed the correct target. This research shows that deer pellets preserved in a technical grade ethanol are still viable for molecular analysis with the correct extraction method.

CONCLUSION

Deer's fecal samples preserved in technical grade ethanol can be viable for further molecular analysis. The surface scrapping method successfully yields better DNA quality compared to the other methods tested and viable for PCR amplification and sequencing. Storage time for up to 2.5 months does not significantly affect DNA concentration and purity. DNA purity is the main factor which affect PCR amplification success. DNA concentration also affect amplification success, although not as significant as DNA purity.

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