Evaluating preservation medium for the storage of DNA in African lion Panthera leo faecal samples

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Abstract  Lion faecal samples, collected in the field between 1 hour to 1 week after defecation were preserved in three different media (ethanol, ASL buffer and Two-step storage). The aim was to determine which faecal DNA field preservation method best enhances PCR amplification success. Samples stored in ethanol showed a significantly higher amplification success of microsatellite loci than samples stored in the other two media. In contrast, amplification success of a mitochondrial locus was similar among the samples stored in the three types of media. We reviewed twelve previous studies that employed different media for the storage of faeces, although patterns of success were not fully consistent among different media, ethanol storage was scored highest in the majority of these tests [Current Zoology 60 (3): 351–358, 2014].

Keywords  Amplification success, Faecal sample DNA, Lion Panthera leo, PCR, Preservation medium

For small and elusive wildlife populations, non-invasive DNA sampling from faecal samples is a feasible method for obtaining genetic data (Kohn and Wayne, 1997). Several studies have employed this method to estimate and monitor wildlife (Kohn et al., 1999; Ernest et al., 2000; Banks et al., 2002, Flagstad et al., 2004, Bensch et al., 2006; Perez et al., 2006; Zhang et al., 2009). For elusive carnivores such as lions Panthera leo non-invasive sampling is especially suitable because faecal samples are easily encountered and recovered along trails where they defecate to mark territory boundaries (Macdonald, 1980). However, studies that employ non-invasive sampling are confronted with pitfalls such as low quantity and quality DNA which can result in low genotyping success and frequent genotyping errors (Taberlet et al., 1997).

The amount and quality of DNA initially present in faeces samples at the time of collection may change during the period of storage and will determine the amount and quality of DNA that can be extracted and used in downstream analyses. It is therefore important to transfer samples into a storage condition that best preserves the DNA prior to the laboratory analysis. The choice of a good preservation medium should be of prime importance for every genetic study, especially when collected samples cannot be processed immediately at the collection site. The identification of effective preservation methods can enhance PCR amplification success rate, increase genotyping accuracy and increase the feasibility of using faecal DNA for genetic studies.

Faecal DNA has so far mainly been used in studies of mammals and less often for studies of birds (Idaghdour et al., 2003; Regnaut et al., 2006). In mammals, DNA has been recovered from faeces that were preserved dried (Höss et al., 1992; Kohn et al., 1995; Foran et al., 1997; Taberlet et al., 1997), in 70% ethanol (Höss, 1992; Kohn et al., 1995), in 99%–100% ethanol (Gerloff et al., 1995) and frozen at -20°C (Reed et al., 1997).

Several studies have evaluated the relative genotyping success from two or more preservation media (Wasser et al., 1997; Frantzen et al., 1998; Murphy et al., 2002; Panasci et al., 2011). These studies span an array of mammal species (Appendix 1) including several species of carnivores (e.g. Wasser et al., 1997; Murphy et al., 2002; Santini et al., 2007; Panasci et al., 2011; Reddy et al., 2012). A few studies comparing storage methods in tropical environments have been conducted mostly on primates (Gerloff et al., 1995; Frantzen et al., 1998; Bayes et al., 2000; Nsubuga et al., 2004; Vallet et al., 2007). Frantzen et al. (1998) attempted to evaluate the success of preserving baboon Papio cynocephalus urainus DNA in four different media and pointed out that the optimal methods are variable according to species and conditions at study sites.

Although some studies have evaluated the relative
success of the same preservation media (Table 1) they have reached different conclusions on their performance. This suggests that effectiveness varies among taxa, environmental conditions and possibly other variables. These conflicting findings call for more studies to be conducted in order to establish the medium that is best for the preservation of faecal samples. In particular, there are relatively few studies from dry tropical regions because most tests have been using fresh faeces (i.e. less than 12 hrs old), therefore we still know relatively little on the storage conditions that are best for the most commonly encountered faeces in the field, i.e. those defecated days to weeks before collection.

In this study we intend to compare the effectiveness of three preservation media, ethanol, ASL buffer and two-step storage (i.e. ethanol and silica gel) for the storage and preservation of lion faecal DNA obtained in Yankari Game Reserve, central North-East Nigeria. The aim is to determine the preservation method that best enhances PCR amplification success of both the mitochondrial and nuclear DNA. This study is part of an ongoing research project that uses DNA from lion faecal samples to monitor lion populations in protected areas in Nigeria (Tende et al., 2010). We hypothesise that the PCR amplification success will differ between the preservatives used. The outcome of our test was evaluated in relation to a literature compilation of other studies testing preservation media.

1 Material and Methods

1.1 Study site
The study was conducted in Yankari Game Reserve (the reserve), central North-East Nigeria (9°50’ N and 10°30’ E), with a landmass of 2,244 km². The reserve lies in the Sudan Savannah zone and the vegetation is composed mainly of dry savannah woodland with a narrow floodplain, bordered by patches of gallery and riparian forests (Geerling, 1973; Crick and Marshall, 1981; Green, 1989). Temperature ranges between 18–24°C during the wet / harmattan season and 30–45°C during the dry hot season.

1.2 Faecal sampling
We conducted daily faeces surveys between January and April 2011 within the reserve, by visiting tracks both in the mornings and evenings. We collected 23 faecal samples of similar age, determined to be between 1hr and 1 week old because we often travelled these tracks more than once per day and at least on a weekly basis. Efforts were made to collect samples that were as fresh as possible (c.f. Piggott, 2004; Murphy et al., 2002). The freshness of faeces was determined based on moisture content, appearance and strength of odour (Vynne et al., 2011). We collected our samples from the outer layer of each faeces, according to Fernando et al. (2003). Each of the twenty-three samples were preserved in the three different media (*n* =69); 95% ethanol, ASL buffer (Qiagen) and Two-step storage (ethanol and silica beads; e.g. Wasser et al., 1997; Reddy et al., 2012). Wherever a sample was encountered a small portion from the outer part of the faeces (c.f. Bidlack et al., 2007; Ferrando et al., 2008; Stenglein et al., 2010) was collected using dry sticks and immediately put into ethanol and ASL buffer. Each stick was discarded after each sample collection to avoid contamination.

| Authors (year) | Climate       | Preservation methods |
|---------------|---------------|----------------------|
| This study    | Tropical, dry | Ethanol 2-step Buffers ASL DETs RNAlater GUS Silica Drying Freezing |
| Reedy et al., 2012 | Temperate, dry | 1 3 2 - - - - - - |
| Roeder et al., 2004 | Temperate | 2 1 - - - - 3 - - |
| Calderon et al., 2009 | Tropical, humid | 2 - - - 1 - - - - |
| Santini et al., 2007 | Temperate, dry | 2 - - - - 1 3 3 |
| Frantz et al., 2003 | Temperate | 1 - - 2 - - - - 3 |
| Piggot & Taylor, 2003 | Temperate | 1 - - 2 - - - - 1 1 |
| Murphy et al., 2002 | Temperate | 1 - - - - 2 3 - |
| Frantzen et al., 1998 | Temperate – Sub-tropical | 1 - - 1 - - - - 2 |
| Wasser et al., 1997 | Temperate | 2 - - - - 1 - 3 |
| Nsubuga et al., 2004 | Tropical, humid | - 1 - 2 2 - 2 - - |
| Panasci et al., 2011 | Temperate, dry | 1 - 2 - - - - |

The figures 1–3 denotes the within study results for preservation, where 1=best, 2= second best, 3= third best.
preserved with the Two-step storage method were first collected in 95% ethanol and kept for 24 hours at ambient temperature. Afterwards the ethanol was carefully poured off and the dry solid faecal sample was transferred into the tube containing silica beads (c.f. Roeder et al., 2004; Reddy et al., 2012). The top of the tube containing the dry silica gel and faecal sample was then carefully stuffed with cotton wool to make it air tight. All collected samples were stored at room temperature, thereafter transported to the laboratory and stored at -33°C prior to DNA extraction.

DNA extraction was carried out in a separate room exclusively dedicated to faeces and hair extractions. DNA from faeces was extracted using the stool DNA extraction protocols in QIAamp® (Qiagen) according to the manufacturer’s instructions. All DNA extractions and PCRs contained a negative control (reagents only) to monitor for contamination at any step along the way.

1.3 Amplification of mitochondrial DNA

In order to evaluate the amplification success of the mitochondrial DNA, all extracted samples were PCR amplified using primers LIHYF (5′-ATGACCAACATTCGAAAATCWC-3′) and LIHYR (5′-ATGTTGGGTSACTGATGAG-3′). These primers are designed to amplify a short portion of the mitochondrial cytochrome b gene (206 bp) and enhance the detection of the target species, while avoiding the amplification of human and ungulate DNA (Tende et al., 2010). All amplifications were carried out using a 2X QiaGen multiplex PCR kit in 10 µl reaction volumes; containing 5 µl Qiagen multiplex PCR buffer mix, 0.2 µM forward primers (Applied Biosystems), 0.2 µM reverse primer, 2.6 µl of water and 2 µl of DNA extract. The PCR profile consisted of the following program: a hot start at 95°C for 15 min; 35 cycles at 90°C for 30 s, annealing of 52°C for 30 s, and an elongation of 72°C for 30 s. A blank control (reagents only) from the extracted DNA process was included in all PCRs to monitor for contamination. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining.

All samples that were successfully amplified were sequenced using the forward primer (BigDye sequencing kit; Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems) for species identification. This is necessary to ensure that the faeces used in the study are from the lion and not from other carnivores such as the spotted hyena Crocuta crocuta and striped hyena Hyaena hyena present in the reserve. Sequences were aligned against reference sequences of lion, spotted hyena and striped hyena obtained from GenBank. All samples assessed to belong to the lion were selected for further analysis.

1.4 Amplification of nuclear DNA

To evaluate amplification success and genotyping accuracy of the nuclear DNA, all positive samples found to originate from lion were PCR amplified using six polymorphic microsatellite primers (FCA001, FCA026, FCA031, FCA077, FCA506 and FCA567; Menotti-Raymond et al., 1999). PCR amplifications were performed in 6 µl multiplex reactions containing 0.12 µl (concentration: 10 µM) dye-labelled (6-Fam or Hex) F-primer, 0.12 µl unlabelled R-primer (concentration: 10 µM), 3 µl of 2X Qiagen Master mix, 0.76 µl double distilled water and 2 µl DNA extract. PCRs were done in a GeneAmp 9700 thermocycler (Applied Biosystems) with the following program: 95°C for 15 min; 40 cycles at 94°C for 30 s, 52°C for 90 s and 72°C for 90 s; followed by an elongation period at 72°C for 10 min. Primers were multiplexed together in two batches based on differences in fragment length and dye. The primer combinations were as follows: FCA001-FCA026, FCA031-FCA567, FCA077-FCA506. Each sample and locus was PCR amplified three times in order to ascertain the results. Alleles of the PCR products were separated using capillary electrophoresis in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Alleles were sized relative to GS500 ROX size standard and proof read and scored in Gene Mapper 4.0 (Applied Biosystems). No allele was accepted unless it was detected at least twice in the three independent PCRs.

1.5 Data analysis

Direct and parallel comparison of the amplification success of the samples obtained from the three different media was made from the genotype result obtained from the six microsatellites, by direct count of the number of successful amplifications. Success rate was then estimated as the number of samples that amplified at a locus from each medium. We tested if the probability of success or failure (1, 0) at the six different loci is dependent on preservation method by building a generalized linear mixed model (GLMM) with a binomial error structure with the probability of success as the response variable, preservation method (Ethanol, ASL buffer or Two-step storage) and loci (1–6) as explanatory variables and sample replicates as a random factor, thus:

\[
\text{Success (1/0) = preservation method (Ethanol, ASL buffer or Two-step storage) + loci (1-6) + replicates (random factor)}
\]

The model also included two-way interactions of the
explanatory variables and using a stepwise backward elimination process, the final model which best explains the variability in the data was selected based on the value of the Akaike Information Criterion (AIC). The best-fit model was the one with the lowest AIC value.

2 Results

Of the 23 samples collected, the cytochrome b gene was successfully amplified in all three preservation methods, except for one sample that was only amplified from the two-way storage medium. There was no apparent difference in the strengths of the bands amplified from the samples in the three different methods of preservation. The sequencing confirmed that in 20 of the samples the amplified cytochrome b gene matched to lion (after aligning our sequences to reference sequences of the lion, spotted and striped hyenas obtained from the GenBank) whereas three samples matched to spotted hyena.

The overall microsatellite amplification success was 50% for samples stored in ethanol, 30% for ASL buffer and 20% for Two-step (n=20 individuals, 6 loci). Ethanol storage showed the highest amplification success at all six individual microsatellite loci and this effect was significant when compared to Two-step storage for five of the six loci (Fig. 1). A logistic regression (Table 2) confirmed the main effect of storage method (P < 0.001) and also showed a significant difference between the loci (P < 0.001). On average, the highest amplification success was obtained at locus FCA077. The non-significant interaction between preservative method and loci (Table 2) indicates that the probability of obtaining a positive outcome at any locus was not influenced by the preservation method used.

3 Discussions

Several studies have evaluated methods for preserving DNA in faecal samples from mammals (Table 1). Although most studies compared some of the different media commonly used, storage in ethanol scored high in the majority of studies where it was tested. This pattern agrees with our study of lions.

We found that mtDNA was amplified with a high success (97%) irrespective of the media. However for nuclear markers (the microsatellites) that are the most valuable markers for studies of individual identification and population comparisons we found the highest success for samples preserved in ethanol. This is suggesting that ethanol (>90%) is an ideal medium for preserving DNA in faecal samples from lions in studies aiming for individual identification, population monitoring and population size estimates. Our findings are thus consistent with Murphy et al. (2002) who found that silica gel beads produced poorer results compared to ethanol, and therefore recommended the use of absolute ethanol for the preservation of faecal sample DNA. They also found that brown bear Ursus arctos samples preserved in DETs buffer performed equally well as samples preserved in high percentage ethanol. Moreover, this study also reported that oven-dried samples produced better results than samples stored in silica beads or preserved by microwave drying. In the present study of lions in Nigeria, the climate is often very hot (up to +45°C) and dry during times when faeces are found. The faeces therefore tend to dry quickly (<24 hrs) thus making it

**Table 2** Logistic regression showing probability of amplification success of lion samples (n = 20) preserved in three different media

|                          | Wald chi-squared | df | P      |
|--------------------------|------------------|----|--------|
| Intercept                | 80.462           | 1  | <0.001 |
| Preservation method      | 41.663           | 2  | <0.001 |
| Locus                    | 27.878           | 5  | <0.001 |
| Preservation method*locus| 11.385           | 10 | 0.328  |
| Replicates               | 1.087            | 6  | 0.982  |

![Fig. 1](image_url) Number of successfully genotyped samples after three repeated independent PCRs from the different medium: ethanol (ETOH), ASL buffer (ASL) and Two-step storage, respectively

Stars indicate pair-wise significant (P < 0.05) differences (Chi-square).
unnecessary to dry the samples before storage.

Panasci et al. (2011) showed that coyote faeces preserved in 95% ethanol and DETs buffer performed equally well and both performed better than samples stored in lysis buffer. Wasser et al. (1997) and Panasci et al. (2011) advised against the use of lysis buffer for the preservation of faecal DNA because it could result in the digestion of ingested hair from consumed prey that thereby could act as a potential source of DNA contamination. Also, it is possible that faeces collected in ASL buffer get lysed if stored at room temperature prior to deposition in the freezer. This might have affected DNA quality in our case, since the collected faeces were not deposited immediately in the freezer. Other preservation methods might be useful for faecal DNA storage depending on storage conditions. For example, Wasser et al. (1997) found silica gel to perform well especially when stored at room temperature.

We reviewed 12 different studies that had evaluated the effects of storage medium for DNA in faecal samples based on amplification success both on mtDNA and nDNA (Appendix 1). Although these studies have not evaluated the same methods a striking pattern is that storage in ethanol (70%–95%) proved to be the best medium in seven out of the twelve studies reviewed (Table 1). The two-step storage was found to be the best in three out of four studies (Table 1). Two buffers (GUS lysis buffer and RNAlater) worked well in the few studies that tested them however; this needs to be investigated further.

Reddy et al. (2012) found that the amount of amplifiable DNA in faeces declined with time. This is probably because environmental conditions (e.g. sun exposure, temperature, humidity) facilitate the degradation of DNA in faeces after deposition (Morin and Woodruff, 1996; Farrell et al., 2000; Goossens et al., 2000; Lucchini et al., 2002; Friedberg, 2003; Nsubuga et al., 2004). The degradation of DNA in faeces appears to be faster during warm compared to cool seasons (Lucchini et al., 2002; Vynne et al., 2011). Wasser et al. (1997) and Vynne et al. (2011) found that this rate of DNA degradation is dependent on both the ambient temperature and humidity. It is therefore vital to recover faeces as quickly as possible (Piggott, 2004; Murphy et al., 2007). Faeces freshness can be determined based on moisture content, appearance and strength of odour (Rutledge et al., 2008; Vynne et al., 2011). Vynne et al. (2011) carried out a study in the Brazilian Cerrado, a seasonally dry tropical environment, to determine factors that cause DNA degradation in faeces of maned wolf *Chrysocyon brachyurus*. They found environmental condition as one of the predictors of the amplification success of mtDNA. Thus, the time interval between scat deposition and collection, as well as sample storage duration can influence the amplification success apart from preservation method (Wasser et al., 1997; Frantzen et al., 1998; Goossens et al., 2000). Although efforts were made during our survey period to recover faeces as soon as possible to minimize exposure to environmental degradation, the samples were not analyzed immediately. Thus it is possible that the duration of sample storage in the freezer before analysis (>1 month) could impact our results. This needs to be investigated further in the same species by analyzing samples of different storage periods.

Previous studies have demonstrated that the specific part of the faeces collected may affect the DNA quality and the amplification success (Rutledge et al., 2008; Gobush et al., 2009; Stenglein et al., 2010; Vynne et al., 2011; Wasser et al., 2011). This is because DNA from faeces is obtained from sloughed epithelial cells of the intestinal lining of the focal species (Albaugh et al., 1992). These cells are often not homogenously distributed in the faeces (Wasser et al., 1997). The different techniques used to address the problem of non-uniform distribution of DNA are: 1) swabbing the surface of the faeces (Lampa et al., 2008; Rutledge et al., 2008; Vynne et al., 2011), 2) scraping the surface (Kohn et al., 1999; Fernando et al., 2000; Livia et al., 2007), 3) washing the surface (Banks et al., 2002; Maudel et al., 2004; Piggott, 2004; Bhagavatula and Singh, 2006; Perez et al., 2006), 4) sampling from the outside (Ferrando et al., 2008; Gobush et al., 2009) or 5) homogenizing the faeces before taking a sample (Wasser et al., 1997; Frantzen et al., 1998; Puechmaille et al., 2007). Samples from the surface of the faeces will thus be more likely to contain the sloughed intestinal cells and yield good DNA quality (Reed et al., 2004; Prugh et al., 2005).

In conclusion, our results are in agreement with most studies (e.g. Santini et al., 2007; Calderon et al., 2009; Reddy et al., 2012) that ethanol is the best method for preservation of DNA. However, it is necessary to point out certain pitfalls associated with the use of ethanol. For example, tubes may leak during transportation when the caps are not properly tightened which may erode/destroy labelling. Also, there may be transportation restrictions by air because ethanol is highly flammable. As mentioned previously, the amplification success in relation to preservation method might vary depending on study species and condition at study sites. We there-
fore advise that multiple storage media are used until a pilot study has helped in deciding the most promising method.

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### Appendix 1 Overview of studies comparing different preservation media for faecal sample DNA

| Authors (Year) | Study species | Climate at study sites | Preservation methods | Relative success / (%) success |
|---------------|---------------|------------------------|----------------------|--------------------------------|
| The present study | Lion *Panthera leo* | Tropical, dry | i) 95% ethanol  
ii) Two-step storage*  
iii) Buffer ASL | Highest (50%)  
Intermediate (20%)  
Lowest (30%) |
| Reddy et al. (2012) | Tiger *Panthera pardus* | Temperate, dry | i) Two-step storage*  
ii) Ethanol  
iii) Silica beads only | Highest  
Intermediate  
Lowest |
| Calderon et al. (2009) | Forest ungulates (*Cephalophus* spp.) | Tropical, humid | i) RNAlater  
ii) 95% Ethanol  
iii) Silica beads only | Highest  
Intermediate  
Lowest |
| Santini et al. (2007) | Wolf *Canis lupus* | Temperate, dry | i) 95% Ethanol at freezer -20º  
ii) GUS lysis buffer**  
iii) Only freezer -20º  
iv) 95% Ethanol at room temperature | Highest (98%)  
Second best (92%)  
Intermediate (71%)  
Lowest (55%) |
| Nsubuga et al. (2004) | Mountain gorilla *Gorilla beringei beringei*  
Chimpanzee *Pan troglodytes versus* | Tropical, humid | i) Two-step storage*  
ii) Silica beads only  
iii) RNAlater solution | Highest  
Intermediate |
| Roeder et al. (2004) | Gorilla *Gorilla gorilla* | Temperate, dry | i) 90% Ethanol  
ii) Two-step storage*  
iii) Silica beads only | Highest  
Second best  
Lowest |
| Frantz et al. (2003) | Eurasian Badger *Meles meles* | Temperate | i) 70% Ethanol  
ii) DETs ***  
iii) Freezing at -20ºC | Highest (89%)  
Intermediate  
Lowest |
| Piggot & Taylor (2003) | Tasmanian pademelon *Thylogale billardieri* | Temperate | i) Freezing at -20ºC  
ii) 70% Ethanol  
iii) Dried at room temperature  
iv) DETs buffer*** | High (55%)  
High (55%)  
High (55%)  
Lowest |
| Murphy et al. (2002) | Brown bear *Ursus arctos* | Temperate | i) 90% Ethanol  
ii) Silica beads only  
iii) Oven-dried (stored at room temperature)  
iv) Oven-dried (stored at -20ºC) | Highest (86%)  
Intermediate  
Lowest |
| Murphy et al. (2000) | Brown bear *Ursus arctos* | Temperate | i) Freeze-drying  
ii) Oven drying  
iii) Silica beads only  
iv) Microwave drying | Highest (98%)  
Second best (95%)  
Lowest |
| Frantzen et al. (1998) | Baboons *Papio cynocephalus ursinus* | Temperate - Subtropical | i) DETs buffer***  
ii) Air-dried at room temperature  
iii) Frozen at -20ºC  
iv) 70% ethanol | Highest (70%)  
Second best (67%)  
Intermediate (60%)  
Lowest |
| Wasser et al. (1997) | American black bear *Ursus americanus*  
Sun bear *Helarctos malayanus* | Temperate, dry | i) Silica beads only  
ii) Ethanol  
iii) Frozen at -20ºC | Highest  
Intermediate  
Lowest |
| Panasci et al. (2011) | Coyote *Canis latrans* | | i) 95% ethanol  
ii) DETs buffer***  
iii) Lysis buffer | High  
High  
Lowest |

*Ethanol and silica gel beads  
**3M guanidine thiocyanate, 50 mM Tris-HCl pH 7.0, 25 mM EDTA pH 8.0, 25% Triton X-100  
***20% DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5 and NaCl to saturation