ONLINE RESOURCES

Suiformes conservation: a study case of strategies for DNA utilization

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Introduction

Lack of suitable biological material for genetic studies in wildlife species can be a bottleneck for studies of conservation, phylogeny, evolution and sustainable management of specific wildlife species. This is due to animal stress during immobilization for sample collection, restricted rules in Zoos and Natural Parks or even physical difficulties in getting samples from living animals. Noninvasive techniques are more often used to obtain samples like hair, faeces and old museum collections, among others. However, the amount and quality of DNA obtained is a major concern and comparison of suitable methodologies must be done for optimization of methodologies for specific purposes.

Suiformes are a large group that represents an important repository of biodiversity and constitutes an excellent group for clarification of the phylogenetic relationships based on the results of comparative genomic approaches. They include several species that constitute an important group for meat production and hunting. Pigs and babirusa, peccaries and hippopotamuses belong to the mammalian order Artiodactyla, the even-toed ungulates, of which there are three major lineages or clades — the Suiformes, Tylopoda and Pecora — each ranked as a suborder. Of these, the Suiformes are the only nonruminants (Groves and Grubb 1993).

Among them Suiformes species possess several million years of distinct evolutionary history and show several morphological size variations, changes in several external and internal structures, different environmental adaptations, a characteristic geographical distribution (Macdonald 1988), and also different karyotype (Adega *et al.* 2006). These

species are dispersed in different regions of the world, such as Africa, central and south America, Southeast Asia and the Philippine islands and Europe (Oliver 1993).

One of the challenges that researchers face is the limitation of DNA stock when there are strong constraints in the availability of tissue samples (Holbrook *et al.* 2005). As such, the whole genome amplification (WGA) technique appeared as a fundamental method for obtaining large amounts of DNA with a huge potential in all fields of genetics (Sun *et al.* 2005). In the WGA technique, the entire genome is replicated leading to large amounts of DNA available (micrograms) from initial quantities of a few nanograms (e.g. Hawken *et al.* 2006) with this amplified DNA being suitable for subsequent sequencing (Dean *et al.* 2001). This technique can also generate large amounts of starting DNA directly from a reduced number of cells, in alternative to extraction and purification methods of DNA (Hosono *et al.* 2003). In the past 12 years, we have witnessed a remarkable evolution in the amplification of gDNA field. Several strategies for amplification have been developed, each one with their advantages and limitations.

There are various methods available to amplify genome, many of them widely studied using human gDNA as a template, but in veterinary research these methods have not yet been highly developed (Short *et al.* 2005). All these methods fall into three main categories: PCR amplification methods, multiple displacement amplification (MDA) methods (used in this work) and OmniPlex® WGA technology (Barker *et al.* 2004; Bergen *et al.* 2005; Park *et al.* 2005; Short *et al.* 2005). MDA differs from other WGA methods, mainly because it is not based on PCR. MDA methods offer the advantage of minimizing existing errors in the other methods, and they ensure that there is no key sequences loss (Dean *et al.* 2002). All the MDA methods are summarized in simple isothermal reactions carried out by the ϕ 29 DNA polymerase (Blanco *et al.* 1989).

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Studies using these innovative MDA methods, to amplify human DNA from various sources, including blood (Dean *et al.* 2002), cell lines (Barker *et al.* 2004), buffy coat, saliva (Hosono *et al.* 2003) and residual cells (Sorensen *et al.* 2004) showed very positive results attesting the impartial amplification of human gDNA.

Materials and methods

Genomic DNA extraction from muscular and fecal tissue, their concentration and quality evaluation

The genomic DNA extraction from muscular tissue (*Sus scrofa domestica* (Ssd); *Sus scrofa scrofa* (Sss); *Pecari tajacu* (Pt) and *Phacochoerus africanus* (Pa)) was performed using (Fujifilm, Tokyo, Japan, Quick-Gene DNA tissue kit S (DS) and the DNA extraction from faecal tissue (*Sus cebifrons cebifrons* (Sec); *Sus barbatus barbatus* (Sbb); *Potamochoerus porcus* (Pp) and *Potamochoerus larvatus koiropotamus* (Plk)) was performed by the commercial kits ZR Fecal DNA Kit™ (Zymo Research, Irvine, USA) and QIAamp® DNA Stool Mini kit (Qiagen, Hilden, Germany). The DNA concentration and purity evaluation were performed by spectrophotometer (NanoDrop ND-1000, Wilmington, USA) and agarose gel electrophoresis.

Whole genome amplification

The whole genome amplification (WGA) technique was applied to the DNA extracted from faeces using GenomiPhi V2 DNA Amplification kit (GE Healthcare, Canada) and REPLI-g® Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The WGA DNA was further compared with genomic DNA.

PCR amplification of a nuclear histone H4 gene fragment and a short interspersed nuclear element (SINE) fragment

Degenerated primers for H4 gene (Pineau *et al.* 2005) and primers from a SINE fragment (Calvo *et al.* 2001) were used to confirm that the DNA was amplifiable after the extraction (muscular tissue) and WGA and extraction strategy (in the faecal samples) (table 1). To obtain the histone H4 gene fragment, we used a 2× Master Mix from Qiagen, Hilden, Germany and a Q solution. To obtain the SINE fragment, we used a 2× Master Mix from Fermentas, Canada.

Results

DNA extraction

The DNA extraction from muscular tissue was more efficient, as we obtained superior yields and high purity levels ($A_{260/280}$), compared to those obtained from faeces. The average yield of DNA extracted from muscular tissue was 32.3 µg, a much higher value than 7.1 µg which was obtained from faeces.

Whole genome amplification

Comparing the two protocols (table 2), it was observed that in the GenomiPhi V2 DNA Amplification kit, from an average 91.1 ng of initial DNA we could obtain an average yield of 15.5 µg amplified DNA, and in the REPLI-g® Mini kit from 182.9 ng of initial DNA an average yield 12.9 µg was reached. Regarding the $A_{260/280}$ ratio almost all samples of amplified DNA (in both kits) had higher values than those of the initial DNA. The $A_{260/280}$ values of initial DNA had an average of 1.5, which increased to 1.8 after the DNA amplification.

PCR amplifications

Figure 1 shows the results of PCR amplification in some of the Suiformes species, for H4 and SINE fragments.

Discussion and conclusion

Suiformes comprise a number of species of great biological interest and economical value. They interact with humans and are domesticated, raised, folded, translocated, hunted, eaten and, in some cases, revered or persecuted. Nevertheless, some Suiformes species biology is poorly known, and their husbandry and strategies for conservation management cannot enjoy the basic information needed. The application of genetic population models and molecular technologies serve the purpose of assisting in the definition and implementation of strategies for preserving biodiversity, considered more appropriate at different levels (genes, populations, species and evolutionary lineages), and may be used to answer countless questions about the biology of Suiformes. Due to the lack of biological material from some wild

Table 1. Primer sequences and amplification conditions.

Primers	Primer sequence	Annealing temperature (°C)	Expected product size
H4F2 (Pineau <i>et al.</i> 2005)	F: 5'-TSCGIGAYAACATYCAGGGIATCAC-3' R: 5'-CKYTTIAGIGCRTAIACCACRTCCAT-3'	57°C	211 bp
SINE (Calvo <i>et al.</i> 2001)	F: 5'-GGATCCGGCATTGCCGTTAG-3' R: 5'-GTCTTTTTTTGCCATTCTTGG-3'	50°C	161 bp

Table 2. DNA quantification before and after the WGA protocol for feces samples.

Samples	Protocol	Before WGA		After WGA		Yield (μg)
		ng/ μL	A _{260/280}	ng/ μL	A _{260/280}	
<i>Sus cebifrons cebifrons</i>	GenomiPhi	56.1	1.5	627.1	1.8	12.5
<i>Potamochoerus porcus</i>	GenomiPhi	126.1	1.4	923.8	1.8	18.5
<i>Sus cebifrons cebifrons</i>	REPLI-g [®]	56.1	1.5	235.1	1.8	11.8
<i>Potamochoerus porcus</i>	REPLI-g [®]	126.1	1.5	392.9	1.9	19.6
<i>Potamochoerus larvatus koiropotamus</i> (1)	REPLI-g [®]	83.0	1.8	181.4	1.8	9.1
<i>Potamochoerus larvatus koiropotamus</i> (2)	REPLI-g [®]	56.6	1.4	238.3	1.8	11.9
<i>Sus barbatus barbatus</i>	REPLI-g [®]	44.0	1.3	245.2	1.8	13.3

Figure 1. PCR products analysed by electrophoresis on 1.5% agarose gel. A, SINE PCR product (≈ 161 bp); M, molecular marker; 1, *Sbb*; 2, *Plk*; 3, *Pp*; 4, *Scs*; 5, *Pt*; 6, *Pa*; 7, *Sss*. B, histone H4 PCR product (≈ 211 bp); 1, *Ssd*; 2, *Sss*; 3, *Pt*; 4, *Pa*; 5, *Plk*; 6, *Pp*; 7, *Sbb*; 8, *Scs* (DNA ladders SM0434 and SM0241, Fermentas). These results were obtained in three independent experiences.

species, in this study we also used faeces (besides the muscular tissue), which is, according to some authors (Machiels *et al.* 2000), a material difficult to handle in terms of DNA extraction.

The muscular tissue DNA extraction presents good results in terms of protocol simplicity, quality and quantity of genomic DNA, allowing its subsequent utilization. However, for the DNA extraction from faeces, the strategy was more laborious. Due to the low concentration and high level of degradation of the DNA, we implemented a WGA strategy that creates a high supplement of DNA. Although the current technologies use small amounts of DNA in genetic research, it is advantageous to develop methodologies that ensure the amplification of genomic DNA with the utmost fidelity.

The WGA method was tested by Short *et al.* (2005), using the GenomiPhi V2 DNA Amplification kit to amplify genomic DNA from blood and buccal swabs. They concluded that there is a high level of concordance between the amplified and nonamplified DNA, through the sequencing and identification of some SNPs. They did not find any allelic differences, and the microsatellite genotyping from the amplified DNA showed a good reproducibility. Hawken *et al.* (2006) found that amplified genomic DNA extracted from bovine semen using the same two kits used in the present work did not show detectable errors and preferred amplifications, thus proving the efficiency of these methods in more complex samples.

In table 2, we could observe that in all amplified samples (in both kits), there was an increased concentration of genomic DNA (we obtained an average yield of $14.2 \mu\text{g}$) and an improvement of the DNA purity observable in the A_{260/280} ratio (the purity ratio increased from 1.5 to 1.8). Comparing our results with those obtained by Short *et al.* (2005), we concluded that the GenomiPhi V2 DNA Amplification kit in our study did not reach the same efficiency level (around $109 \mu\text{g}$). Hawken *et al.* (2006) showed superior yield compared to those obtained in the present work, using both methodologies. This fact may be due to the low quality and long storage period of the samples, as reported by Short *et al.* (2005).

Comparing the efficiency of the two WGA protocols obtained in the present work, we observed that the GenomiPhi V2 DNA Amplification kit gives higher return than that of REPLI-g[®] Mini kit, and uses a smaller initial DNA quantity (we obtained an average yield of $15.5 \mu\text{g}$ with GenomiPhi and $13.1 \mu\text{g}$ with REPLI-g). Hawken *et al.* (2006) obtained better results using the REPLI-g[®] Mini kit, contrarily to what we observed. In our case GenomiPhi V2 DNA Amplification kit methodology is shown as preferred, as it used lower initial average concentration, and obtained higher average yields.

To confirm that the DNA extracted from muscular tissue and the DNA extracted from faeces combined with the WGA technique were successful strategies, degenerated primers for

H4 gene (Pineau et al. 2005) and primers for a SINE fragment (Calvo et al. 2001) were used. Pineau et al. (2005) presented a method to control the quality of DNA using the amplification of histone *H4* gene with degenerated primers. Calvo et al. (2001) designed specific primers from a fragment with high homology with SINE sequence presented in *S. scrofa*. As such, we concluded that this fragment could also be present in the Suiformes species under study (given their homology), and due to its reduced size (161 bp) this fragment could be another good candidate to confirm that the DNA extracted and amplified was ready to be used in subsequent molecular analysis. As we expected, amplification of histone *H4* gene and *S. scrofa* SINE sequence in all DNA samples (extracted from muscle and faecal tissue) was achieved (figure 1), confirming again the DNA quality for the subsequent molecular studies.

The amount of DNA available for research is often insufficient, so it is necessary to use genome amplification kits. With the help of these kits, it is possible to obtain almost unlimited source of DNA. This work showed that the use of WGA technique in this type of situation is of great interest, because it allowed the profitability of the existing samples, improving in addition the DNA purity. This strategy, applied to Suiformes species can be very valuable in future genetic research in order to provide adequate DNA supplies of endangered wildlife species.

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