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# COMPARATIVE DNA DEGRADATION IN BONES FRAGMENTS

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**Abstract:**

*The aim of this study is to follow the changes that occur, in time, at DNA level and to establish an efficient and reliable protocol for ancestral DNA extraction from bones found in archaeological sites. To test whether the protocol is efficient and capable of yielding good quality DNA, extraction was first performed on fresh bones. The material consists of fresh pig (Sus scrofa) and cow (Bos taurus) bones that were grounded by using a drill operating at low speed and the bone powder was incubated in lysis buffer in the presence of proteinase K. DNA isolation and purification were done by using the phenol: chloroform protocol and DNA was precipitated with absolute ethanol stored at -20°C. The extractions were carried out once every month for a total of four extractions.*

**Keywords**: *DNA, bone tissue, ancient DNA, total DNA.*

## Introduction

Higuchi et al. managed to isolate, amplify and sequence fragments of DNA from an animal that had been extinct for 100 years. DNA was recovered from dried out muscle tissue that belonged to a museum specimen of the quagga (*Equus quagga*). When the first piece of ancient DNA was sequenced (Higuchi et al. 1984), it set the basis of a new research field in genetics and paved the way for many scientists. The instances of soft tissue preservation are rare and the integrity of DNA can depend on a variety of factors. When dealing with ancient DNA, bones are the preferred means (and sometimes, the only means). Soft tissues are rapidly decomposed but bones can withstand the environmental conditions and part of the initial DNA evades degradation. Soft tissue can also be preserved but under special circumstances like rapid dehydration or mummification (Sonoda et al. 2000; Konomi, Lebwohl, and Zhang 2002; Ghanem et al. 2005) and freezing (Ermini et al. 2008; Willerslev, Hansen, and Poinar 2004; H. N. Poinar 2006; Miller et al. 2008).

When soft tissue is unavailable, DNA extraction is carried out on bone remains. Depending on the conditions in which bone material is preserved, viable DNA fragments can be recovered from bones and/or teeth up to several thousand years old (Rohland and Hofreiter 2007; Adler et al. 2010). DNA quality depends mostly on the type of soil

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the biological material is buried in and other factors like temperature, humidity and sun exposure (Alaeddini, Walsh, and Abbas 2010; Kaiser et al. 2008). If the bone is kept at high humidity and temperature levels, DNA damage is likely to occur short time after cell death. Besides chemical alterations like hydrolysis and oxidation (Lindahl 1993), postmortem DNA is liable of physical modifications inflicted by microorganisms, soil invertebrates and cellular nucleases (Binladen 2005).

The aim was to test whether the extraction protocol that we used on modern bones would yield good quality DNA from bones.

**Materials and Methods**

*DNA extraction*

The material consisted of fresh pig (*Sus scrofa*) and cow (*Bos taurus*) bones. The first total DNA extraction was carried out in the moment the bones were acquired, after which the extractions were done once every month, for a total of four extractions.

Bone fragmentation was accomplished by using a drill operating at low speed. The bone surface was properly cleaned, using a commercially available 5% sodium hypochlorite solution (Kemp and Smith 2005), the superficial layer of bone was removed and the bone was exposed to UV light prior to DNA extraction to avoid contamination. The bone powder was incubated for 2 hours at 37$^{o}$C in lysis buffer (50mM Tris-HCl *p*H=8, 25mM EDTA, 1% SDS, 20 mg/ml proteinase K) and total DNA extraction was performed using the phenol:chloroform:isoamylic alcohol (25:24:1) protocol. To remove glycoproteins, a 5M NaCl solution was added to the lysis buffer-bone powder mix after the incubation. DNA elution was done in Tris-EDTA. Total DNA quantitation was spectrophotometrically determined and its molecular weight and integrity were determined by 1% agarose gel electrophoresis stained with ethidium bromide and UV light visualized.

*DNA amplification*

To test whether the extracted DNA is of good quality and capable of amplification, it was subjected to a polymerase chain reaction (PCR). The first part of the cytochrome b – encoding gene was chosen for amplification, using the L14109 (5'-CAGGACCAATGACATGAAAAA-3') and the H14743 (5'-GCTGCGAGGGCGGTAAT-3') primers (Alves et al. 2003). The cytochrome b gene is the preferred gene for use in molecular taxonomy studies alongside other mitochondrial protein-encoding genes and the non-coding mitochondrial control region or D-loop. The PCR was conducted in a 25µl reaction volume consisting of 1X GoTaq® Green Master Mix (Promega, Madison USA), 0.2µM each primer, DNA template and nuclease-free water to 25 µl.

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Amplification conditions consisted of an initial denaturation at 95°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute. A final extension step of 10 minutes at 72°C was performed after the 40 cycles. The PCR products were run on a 1% agarose gel stained with ethidium bromide and UV-light visualized.

**Results**

*DNA electrophoresis*

About 100 mg of bone powder from two fresh pig (*Sus scrofa*) and three cow (*Bos taurus*) bones were used for each extraction and the total DNA was subjected to an electrophoresis for a total of four gel runs (*Figures 1* to *4*).

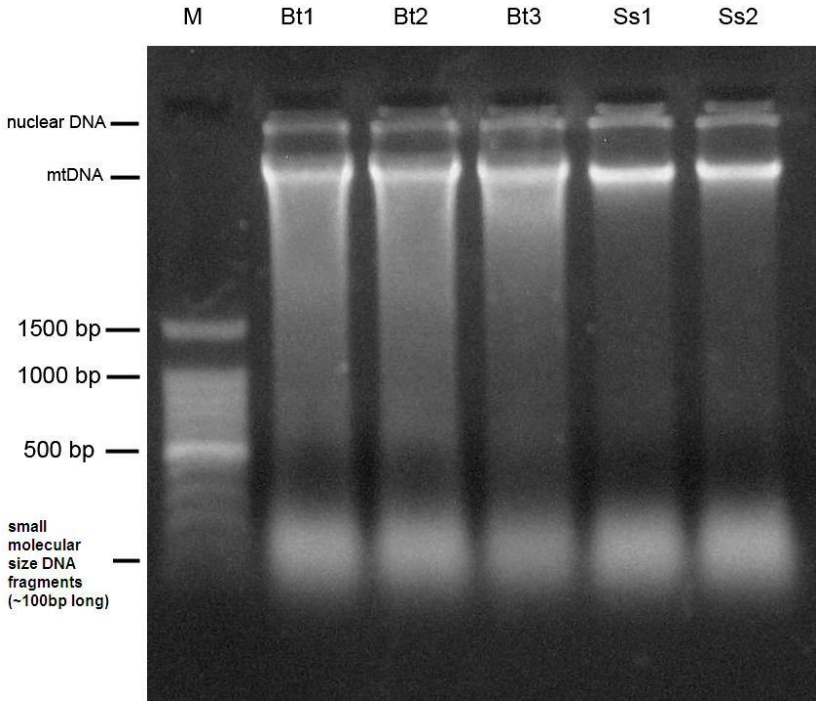


***Figure 1***. Total DNA electrophoresis on a 1% agarose gel for the first extraction.

Lane 1 – 100bp step ladder, Lanes 2-4 – *Bos taurus* total DNA, Lanes 5, 6 - *Sus scrofa* total DNA. The first band from the top represents the nuclear DNA, the second band represents the mitochondrial DNA and the third band represents the small molecular size DNA fragments, which are approximately 100bp-long.

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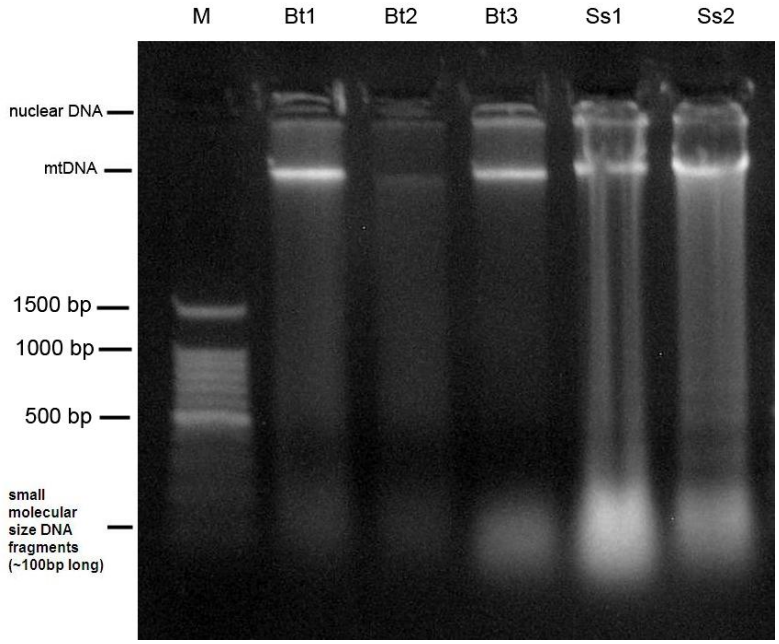


***Figure 2***. Total DNA electrophoresis on a 1% agarose gel for the second.extraction.

Lane 1 – 100bp step ladder, Lanes 2-4 – *Bos taurus* total DNA, Lanes 5, 6 - *Sus scrofa* total DNA. The first band from the top represents the nuclear DNA, the second band represents the mitochondrial DNA and the third band represents the small molecular size DNA fragments, which are approximately 100bp-long

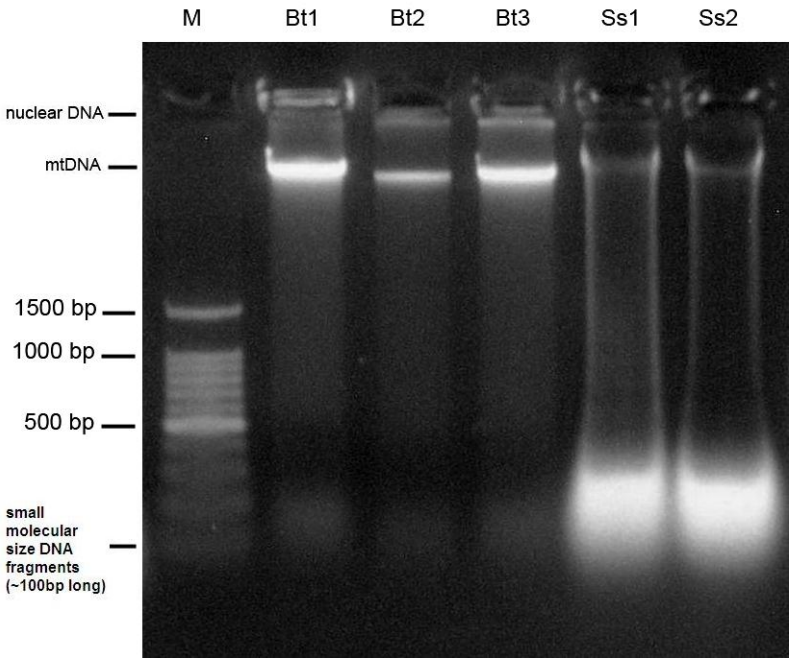


***Figure 3***. Total DNA electrophoresis on a 1% agarose gel for the third extraction.

Lane 1 – 100bp step ladder, Lanes 2-4 – *Bos taurus* total DNA, Lanes 5, 6 - *Sus scrofa* total DNA. The first band from the top represents the nuclear DNA, the second band represents the mitochondrial DNA and the third band represents the small molecular size DNA fragments, which are approximately 100bp-long.

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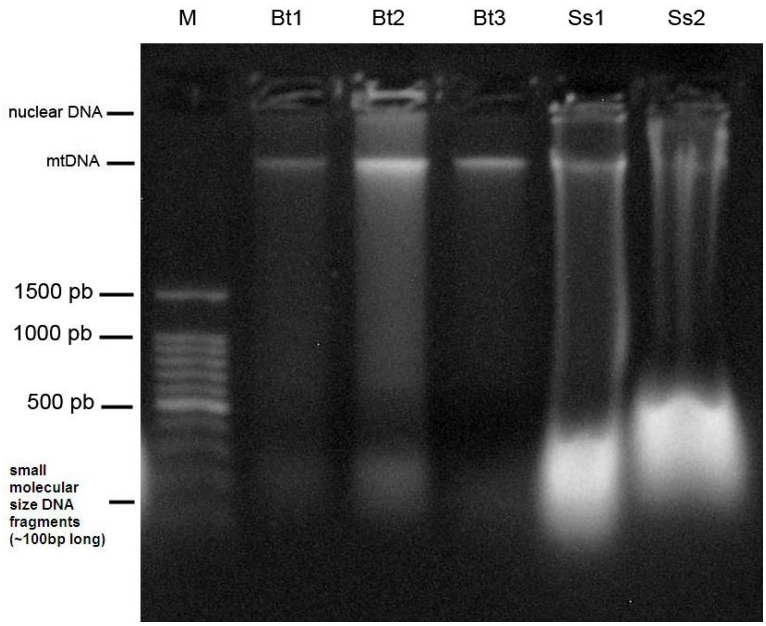


***Figure 4***. Total DNA electrophoresis on a 1% agarose gel for the fourth extraction.

Lane 1 – 100bp step ladder, Lanes 2-4 – *Bos taurus* total DNA, Lanes 5, 6 - *Sus scrofa* total DNA. The first band from the top represents the nuclear DNA, the second band represents the mitochondrial DNA and the third band represents the small molecular size DNA fragments, which are approximately 100bp-long

After all four extractions were carried out, we test whether the isolated DNA was capable of amplification so it was subjected to a PCR.

The first part of the cytochrome b-encoding gene was targeted and the PCR products were run on a 1% agarose gel (*Figure 5*).

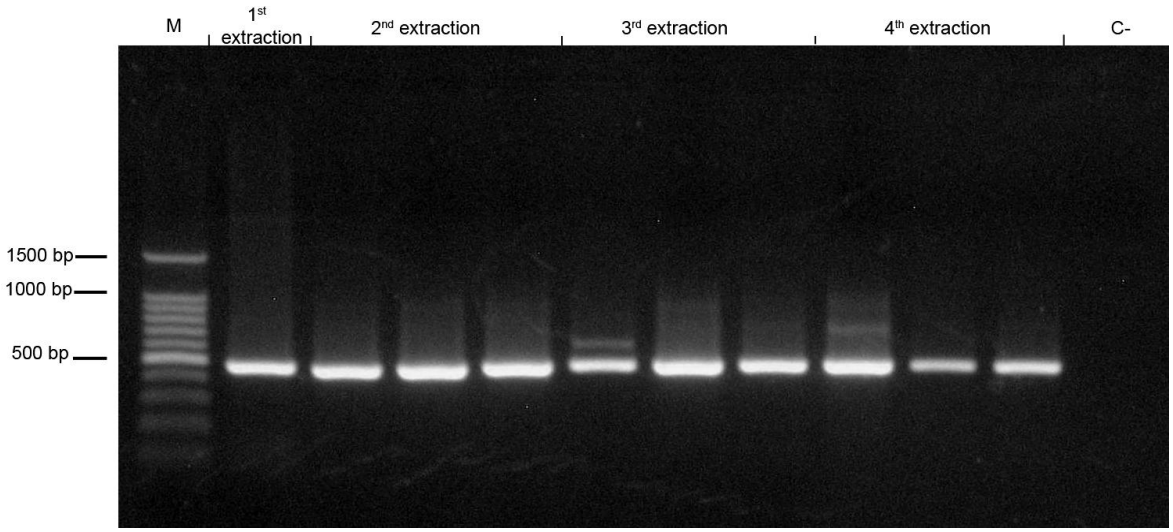


***Figure 5***. PCR products for the first part of the cytochrome b-encoding gene for the *Bos taurus* DNA.

Lane 1 – 100bp step ladder, Lane 2 – PCR product for the first extraction, Lanes 3, 4, 5 – PCR products for the second extraction, Lanes 6, 7, 8 – PCR products for the third extraction, Lanes 9, 10, 11 – PCR products for the fourth extraction, Lane 12 – negative control.

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*Gel analysis*

Gel analysis was performed by the TotalLab Cuant software (TotalLab Ltd., Newcastle upon Tyne, UK). The software calculated the band intensity and percentage and generated the Rf values for each band for all of the four extractions.

The retention factor (Rf) represents the ratio of the distance the band of interest moved from the origin to the distance the solvent front moved. The Rf values are always between 0 and 1. The closer the Rf value is to 1, the smaller the DNA fragment is. Knowing the fact that the smaller DNA fragments move faster than the larger ones, they will have a higher Rf value. Figure 6 shows a graph in which all of the Rf values for each of the bands are included.

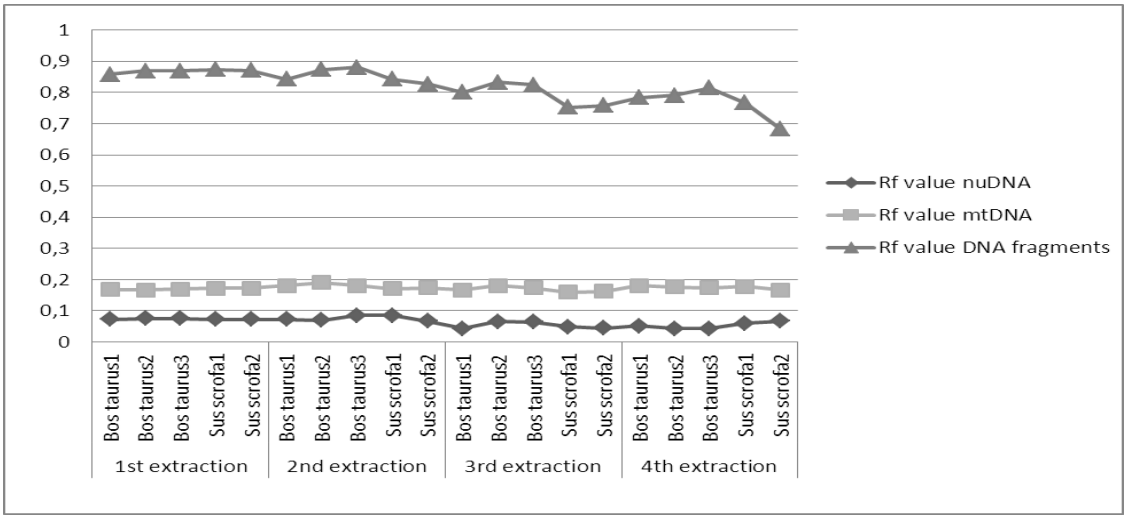


***Figure 6***. Rf values for all bands from all four extractions.

nuDNA – nuclear DNA (high molecular weight), mtDNA – mitochondrial DNA and low molecular weight DNA represented by fragments.

Depending on the intensity and surface of each band, the TotalLab Cuant software was able to calculate the volume percentage of each band.

Figures 7, 8, 9, 10 show the percentage of the averages for each species from the 1st, 2nd, 3rd and 4th extraction, respectively. Considering the fact that the bands for the nuclear DNA, mitochondrial DNA and the DNA fragments have, basically, the same length in-between samples we decided to use the average value for them. So, for each extraction, an average value was calculated for the three samples of *Bos taurus* and the two samples of *Sus scrofa*.

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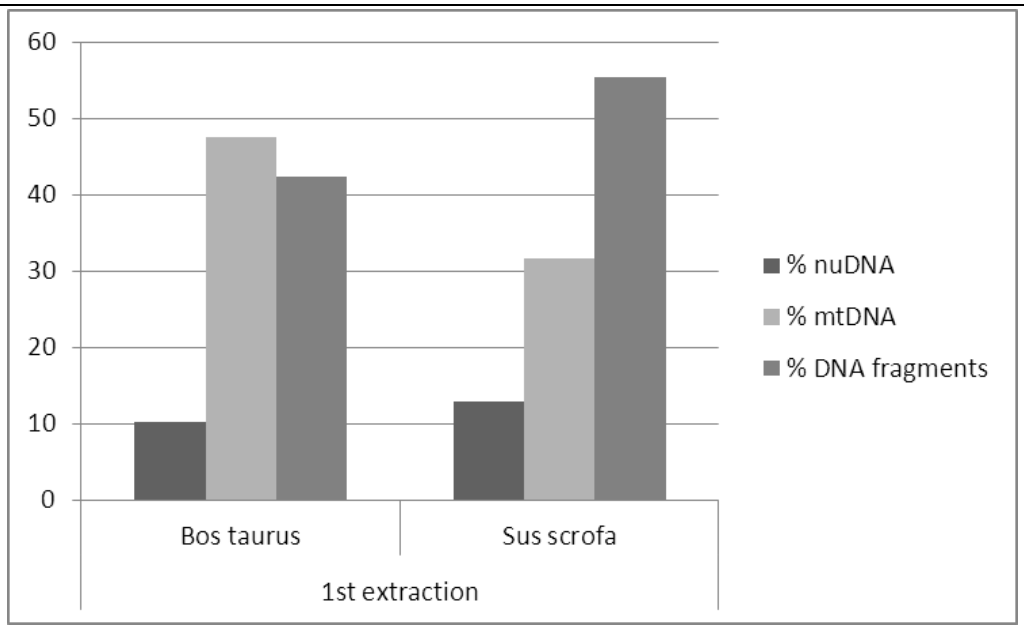


***Figure 7***. Size percent for the averages of each of the bands (nuDNA – nuclear DNA, mtDNA – mitochondrial DNA and DNA fragments) for the two species under investigation for the 1st extraction.

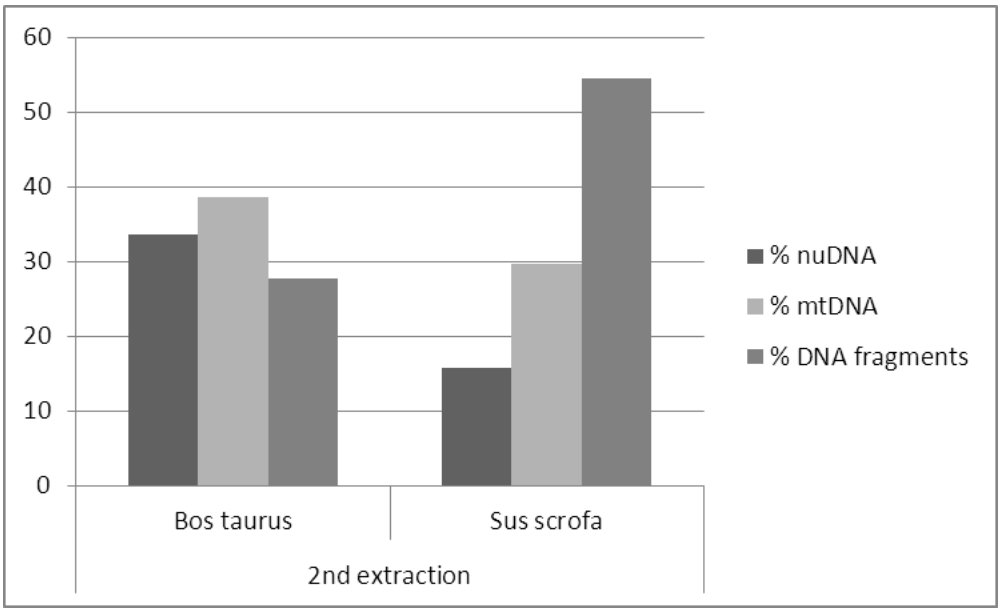


***Figure 8***. Size percent for the averages of each of the bands (nuDNA – nuclear DNA, mtDNA – mitochondrial DNA and DNA fragments) for the two species under investigation for the 2nd extraction.

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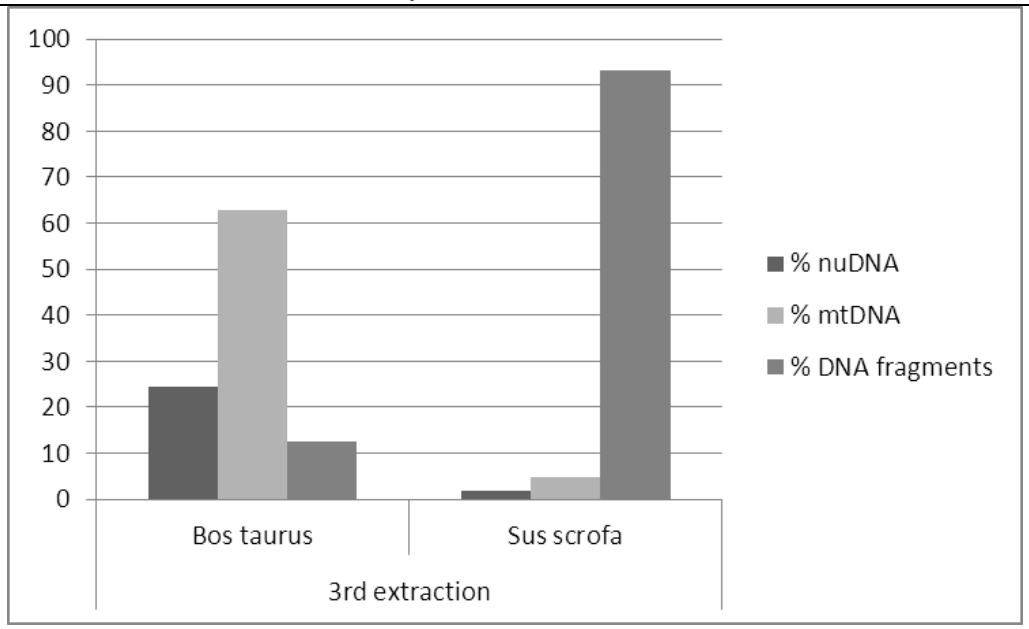


***Figure 9***. Size percent for the averages of each of the bands (nuDNA – nuclear DNA, mtDNA – mitochondrial DNA and DNA fragments) for the two species under investigation for the 3rd extraction.

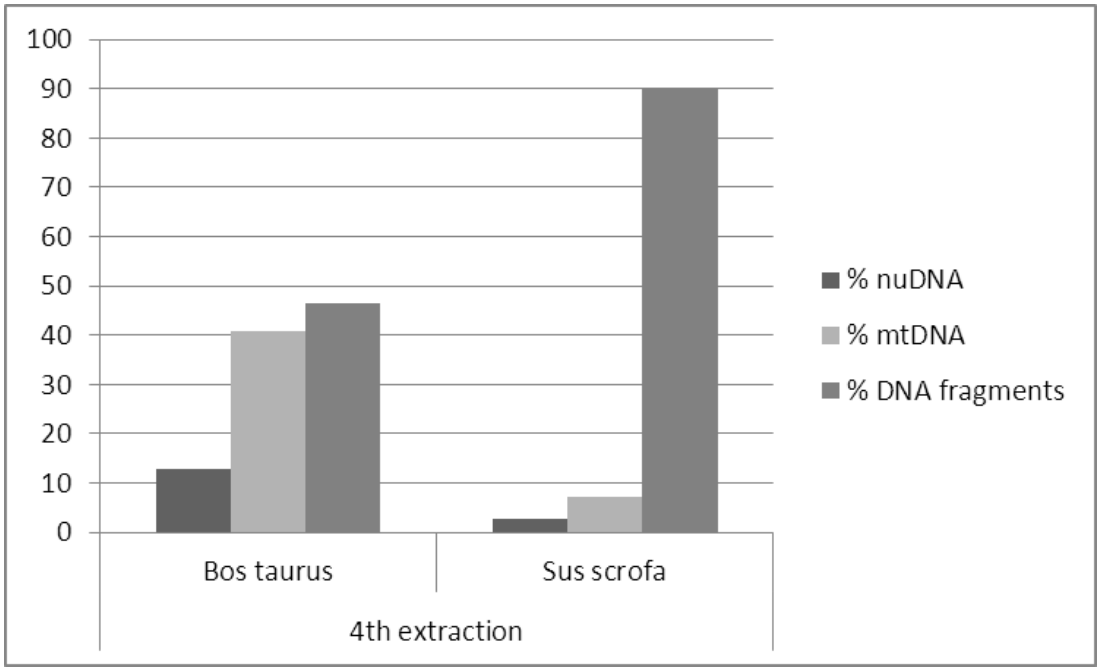


***Figure 10***. Size percent for the averages of each of the bands (nuDNA – nuclear DNA, mtDNA – mitochondrial DNA and DNA fragments) for the two species under investigation for the 4th extraction.

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**Discussions**

High molecular weight DNA (nuclear DNA) is rapidly degraded by endogenous exonucleases once the nucleus envelope is broken (Graham 2007), fragmentation occurring short time after cell death. However, this fragmentation can be delayed or rushed by several factors, like temperature and humidity (Burger et al. 1999). There have been instances in which nuclear DNA was recovered from bone remains (Greenwood et al. 1999; Miller et al. 2008). On the other hand, mitochondrial DNA can maintain its integrity for a long time due to the fact that the mitochondrion genome is considerably smaller and that mtDNA exists in hundreds of copies per cell.

PCR was carried out on all extractions and a fragment of mtDNA was successfully amplified (Figure 5) thus showing that mtDNA, due to its properties mentioned above, is reliable over long periods of time.

This study showed a gradual fragmentation of total DNA indicating a reduction of high molecular weight DNA and an increase of smaller size DNA fragments. These fragments can be seen on the agarose gel as vertical bands known as smearing.

Figure 6 shows a decrease of the Rf value for the smaller-size DNA fragments for the last two extractions, one explanation possibly being the size and number of the fragments themselves. Being in such high number and having great mobility, they could get in each other's way causing a "traffic jam", thus reducing the Rf value.

A difference of DNA integrity between total DNA extracted from the epiphysis and the diaphysis of the bone has been observed. The differences between the two analyzed species can easily be seen in Figures 2, 3 and 4, Sus scrofa DNA fragmentation being clearly more obvious. This is probably because the epiphysis retains most of its water and thus encourages DNA degradation while the diaphysis is more compact and does not contain as much water, thus the humidity factor is relatively low. This leads to a better preservation of DNA.

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