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Isolation of DNA from bone material of selected animals from *Cervidae* family

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ABSTRACT

Bone material is one of the most difficult biological samples used for extraction of DNA. The primary objective of molecular tests is to determine the concentration and quality of DNA, and these results are of significant importance in further analyses. The aim of the study was to evaluate the usefulness of a method for DNA isolation from bone material of selected animal species from *Cervidae* family using a commercially available GeneMatrix Bone DNA Purification Kit manufactured by Eurx. Quantitative evaluation was performed with a spectrophotometer. Pure DNA isolates were obtained from 21 out of 24 bone fragments. The efficiency of the applied isolation method varied. The difference between the lowest and highest DNA concentration was more than hundredfold. Qualitative analysis was carried out by means of electrophoresis in 1% agarose gel. No high molecular mass DNA was obtained. The genetic material was present in small quantity and it was fragmented. It was concluded that the Bone DNA Purification Kit manufactured by Eurx may be effective only in isolation of DNA from bones stored in good conditions.

***Keywords*:** DNA isolation, bone material

1. INTRODUCTION

The quality of DNA isolates constitutes one of key factors affecting all subsequent stages of molecular analyses. Many researchers seek methods that reduce the risk of contamination and yield high quality samples for genetic tests. For this reason, various DNA

isolation and purification methods are used in laboratories. Selection of an adequate DNA isolation method depends on availability of equipment, reagents, type of tissue and intended application of the isolated nucleic acid. A new field of forensic science has started to develop recently, i.e. wildlife forensics that provides tools that help identify DNA of animal origin. Forensic laboratories typically do not carry out genetic tests with animal DNA, since related methods have not yet been standardized as it is the case with human genetic material (Dębska 2013).

Isolation of DNA from problematic materials, such as feathers, hair, bristle or bone poses a challenge for some molecular biology researchers, as they come across several problems. Those, among others, include degradation of DNA. Therefore, it seems that a need arises for development of procedures that make handling of such materials easier (Alaeddini 2010).

Bone tissue is one of the most difficult biological samples used for extraction of DNA. Working with such materials is both time- and labor-consuming (Alonso et al. 2001). The difficulty primarily results from preparation of the sample before the extraction of deoxyribonucleic acid. Typical procedures include removal of contamination, fragmentation and breakdown of bone in a blender or grinder to obtain pulverized material (Davoren et al. 2007). Also, drilling spoil from bones may be used (e.g. from inside teeth) and, if other fragments are available, the tissue may be broken down mechanically and pulped in liquid nitrogen (Słomski 2014). Afterwards, supernatant from the bone powder incubated in lysis buffer solution is collected. Contrary to that, newer procedures are based on the use of entire demineralized bones which increases the efficiency of DNA extraction (Loreille et al. 2007). Organic methods or methods based on silica beds are commonly used in the forensic science (Davoren et al. 2007). The next stage of the procedure is the determination of concentration and purity of the obtained DNA isolates. This may be verified by electrophoresis in agarose gel followed by determination of the quantity and quality of DNA in a transilluminator excited with light of 312 nm wavelength. High molecule mass DNA is obtained when a dense band of 50 kbp is visible. Potential smears typically indicate degraded material (Słomski et al. 2014).

The primary aim of this study was to assess the usefulness of the method for DNA isolation from bone material in selected animal species belonging to *Cervidae* family.

2. MATERIAL AND METHODS

The study material (Figure 1-4) consisted of selected parts of skeleton (mandible or skull bones) of three representatives from *Cervidae* family: red deer, roe deer and fallow deer.

The bone material (denoted with letter K) was sourced from various parts of the West Pomerania province (Poland), i.e. from the following localities:

- Stara Korytnica (53° 18’ N; 16° 2’ E) – bones: K1 to K18,
- Karwowo (52° 13’ N; 21°0’ E) – bones: K19 to K23
- Dorowo (53° 43’ N; 15°27’ E) – bone: K24.

Figure 1. Bone material used for DNA isolation – K1 - K8.

Figure 2. Bone material used for DNA isolation – samples K9 - K18.

Figure 3. Bone material used for DNA isolation – K19 - K23.

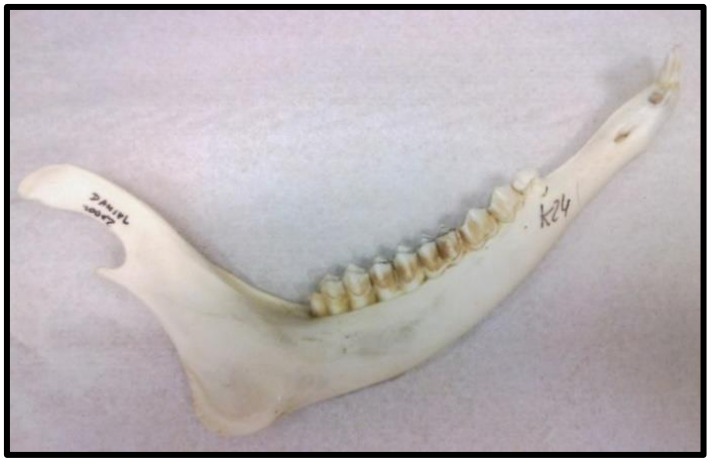


Figure 4. Bone material used for DNA isolation – K24.

DNA isolation consisted of two stages:

- preparation of the study material
- proper DNA isolation

Preparation of bone powder for DNA isolation (Figure 5) consisted in breaking down bone fragments with a Makita drill. Pre-disinfected 3.4 and 3.3 mm Bosch drill bits were used. Below, the disinfection procedure is presented:

- 5% calcium hypochlorite solution was prepared
- The drill bits were soaked in $Ca(OCl)_2$ solution for 10 minutes
- Afterwards, the drill bits were rinsed three times with distilled water
- Subsequently, the drill bits were immersed in 70% ethanol solution for five minutes.
- Immediately before drilling, each bone was wiped with 70% ethanol.

NOTE: The drill bits were replaced for each bone powder extraction operation.
The table below presents the study material with indication of sample symbol, bone name and weight of the obtained powder (Table 1).

Table 1. List of the study materials
(symbol, species, bone type and weight of powder used for DNA isolation).

Symbol	Species	Bone name	Weight (g)
K1	Red deer	Mandible	0.28
K2	Red deer	Mandible	0.26
K3	Red deer	Mandible	0.26
K4	Red deer	Mandible	0.38
K5	Roe deer	Mandible	0.36

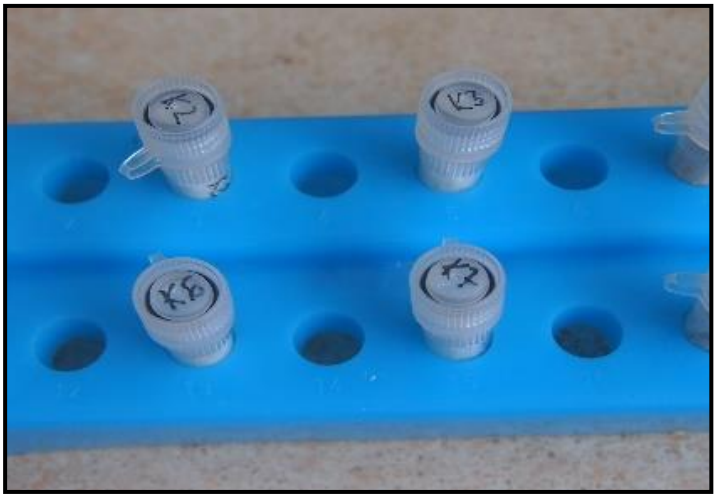
K6	Roe deer	Mandible	0.34
K7	Fallow deer	Mandible	0.33
K8	Roe deer	Frontal bone	0.28
K9	Red deer	Mandible	0.35
K10	Red deer	Mandible	0.24
K11	Roe deer	Frontal bone	0.27
K12	Roe deer	Frontal bone	0.30
K13	Roe deer	Frontal bone	0.22
K14	Roe deer	Frontal bone	0.28
K15	Roe deer	Frontal bone	0.17
K16	Roe deer	Frontal bone	0.38
K17	Roe deer	Frontal bone	0.33
K18	Roe deer	Frontal bone	0.39
K19	Red deer	Mandible	0.24
K20	Red deer	Mandible	0.32
K21	Roe deer	Mandible	0.38
K22	Roe deer	Frontal bone	0.30
K23	Roe deer	Frontal bone	0.30
K24	Fallow deer	Mandible	0.26

Stage 1. Preparation of solutions for disinfection of drill bits

Stage 2. Removal of impurities from bone surface

Stage 3. Several rounds of drilling

Stage 4. Weighing of retrieved bone powder



Stage 5. Bone powder ready for DNA isolation

Figure 5 (Stages 1-5). Preparation of bone powder for DNA isolation.

The isolation of DNA from the bone material was carried out using a column-based method and the GeneMatrix Bond DNA Purification Kit manufactured by Eurx. The manufacturer's procedure was applied as the study methodology (URL 1). Purity and concentration of DNA from the bone material were determined with NanoDrop 2000c spectrophotometer manufactured by Thermo Scientific.

DNA isolates were separated in 1% agarose gel. Visualization of the separated isolates was carried out using G-box transilluminator manufactured by Syngene and compatible GeneSys v. 1.3.5.0 software.

The results were then analyzed statistically. Calculations were carried out using Statistica PL software (version 10). The statistical analysis comprised descriptive statistics and correlation between DNA concentration, absorbance coefficient at 260 and 280 nm wavelength and weight of the obtained bone powder.

3. RESULTS

The DNA from the bone fragments was isolated by column-based method with the use of GeneMatrix Bone DNA Purification Kit manufactured by Eurx. The DNA concentration and purity were evaluated using a spectrophotometer. The results are presented in Table 2, while the statistical analysis is shown in Table 3. Further characterized measures had following values: 1251.7 ng/µl for range and 107.98 for coefficient of variation. These results showed the degree to which the distribution of the examined traits varied. Also, a high value of standard deviation was determined – 312.2 (Table 3). The absorbance coefficient for 260 and 280 nm (so-called A260/A280 ratio) varied between 1.81 and 2.3 with range equal to 0.5. On average, it equaled 1.9. The value of standard deviation (0.12) and coefficient of variability (6.51) were found to be relatively low (Table 3).

Table 2. Results of DNA isolation from bone material using the GeneMatrix Bone DNA Purification Kit manufactured by Eurx.

	DNA concentration (ng/µl)	A260/A280 ratio
K1	216.8	1.95
K2	386.0	1.85
K3	91.1	2.01
K4	180.2	1.87
K5	1264.2	1.82
K6	661.8	1.83
K7	353.7	1.83
K8	187.8	1.85
K9	142.8	1.85
K10	12.9	2.33
K11	23.9	1.88
K12	65.1	1.9
K13	12.5	2.21
K14	474.0	1.85
K15	242.3	1.88
K16	289.4	1.86
K17	51.1	1.86
K18	40.9	1.84
K19	125.5	1.91
K20	259.8	1.83
K21	782.9	1.81
K22	123.2	1.87
K23	832.0	1.83
K24	119.1	1.97

DNA concentration in the tested samples ranged from 12.5 ng/µl to 1264.2 ng/µl with a mean of 289.13 ng/µl.

Table 3. Descriptive statistics calculated in Statistica v.10 software.

	Mean	**Minimum**	**Maximum**	**Range**	**Standard deviation**	**Coefficient of variation**
DNA concentration (ng/µl)	289.13	12.50	1264.2	1251.7	312.20	107.98
A260/A280 ratio	1.90	1.81	2.3	0.5	0.12	6.51

The analysis of the correlation coefficient matrix defining the relationships between examined traits showed little variability. Statistically significant results at $p<0.05$ were obtained for A260/280 ratio and DNA concentration (-0.44) as well as for sample weight and A260/280 ratio (-0.52). In practical terms, it means that in both cases, a moderate correlation existed between the investigated traits, i.e. with increasing A260/A280 ratio, the DNA concentration in the analyzed samples decreased, and with increasing bone powder weight, the value of A260/A280 decreased. The detailed summary is presented in Table 4.

Table 4. Analysis of correlation (the indicated correlation coefficients are statistically significant at $p < .05$ N = 24).

	DNA concentration (ng/µl)	**A260/A280 ratio**	**Weight (g)**
DNA concentration (ng/µl)	1.00		
A260/A280 ratio	-0.44	1.00	
Weight (g)	0.34	-0.52	1.00

The analyzed samples were also electrophoresed in 1% agarose gel. DNA smears appeared in all 24 samples sourced from bone fragments. The visualization of results and their description are presented in Figure 6.

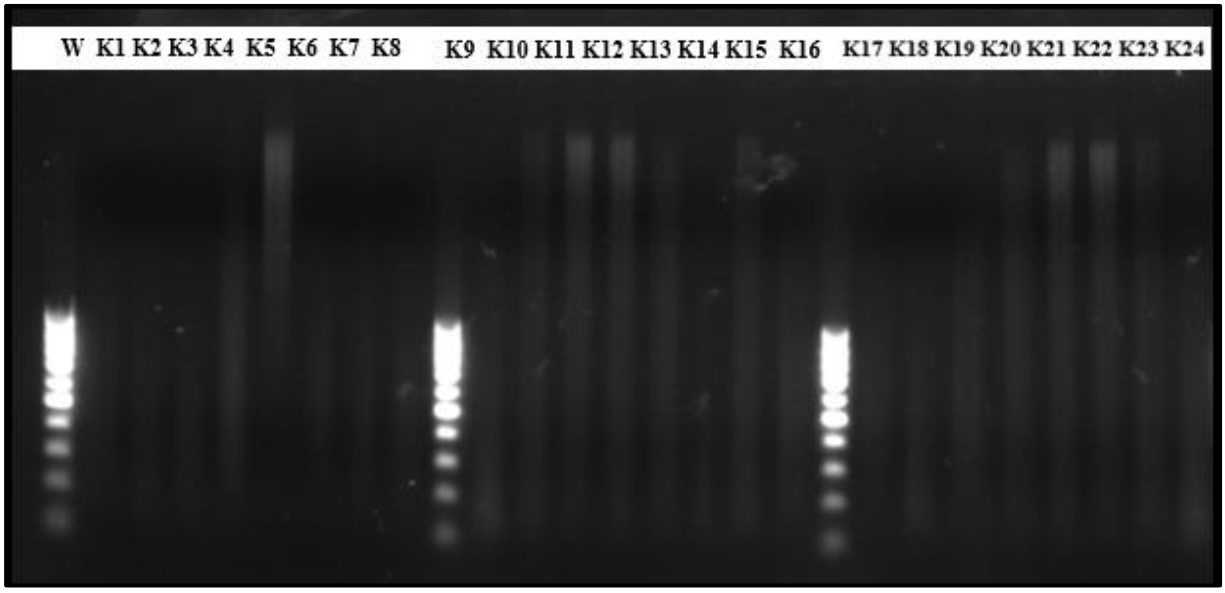


Figure 6. Qualitative evaluation of genomic DNA. Lanes 1, 9, 18 – molecular weight marker - Thermo Scientific MassRuler DNA Ladder Mix. Lanes 2-8 (K1-K8), 10-17 (K9-K16), 19-26 (K17-K24) – genomic DNA sourced from bone material, detailed symbols K1-K4, K9-K10, K20 – red deer; K5-K6, K8, K11-K18, K21 – roe deer; K7, K24 – fallow deer.

4. DISCUSSION

The absorbance ratio for 260 nm and 280 nm provides information on the isolated genetic material. The concentration of nucleic acids is determined by spectrophotometric measurement of the quantity of UV light absorbed by DNA nucleobases and derived from A260/A280 ratio. For pure DNA this ratio equals 1.8. Its value exceeding 2.0 indicates RNA presence in the sample. Further, the value of A260/A280 ratio below 1.8 means that the isolate contains protein or phenol impurities. The DNA suitable for analyses has A260/A280 ratio between 1.8 and 2.0 (Słomski 2014).

In this study, the quantitative evaluation was performed with a spectrophotometer and pure DNA isolates, i.e. with the values ranging from 1.8 to 2.0, were obtained for 21 bone fragments. Among the studied samples, the values exceeding 2.0 were obtained for two fragments of red deer bone marked K3 (2.01) and K10 (2.33) and for a fragment of roe deer frontal bone marked K13. Their value of A260/A280 ratio equaled 2.21. Therefore, the isolates K10 and K13 were contaminated with RNA. The GeneMatrix Bone DNA Purification Kit manufactured by Eurx did not contain ribonuclease, an enzyme that efficiently removes ribonucleic acid from aqueous solutions (URL 1). RNA contamination in these two samples could not be avoided. Sample K3 was considered suitable for further tests, despite slightly exceeded (by 0.01) value of absorbance coefficient.

The efficiency of the applied isolation method varied. The resulting DNA concentration values demonstrated over hundredfold difference between the lowest and highest concentration. The study revealed that one of the most important moments during the DNA extraction process was the preliminary preparation of the bone material. This assumption was

confirmed by other researchers (Słomski et al. 2014), who highlighted the manner of sample storage. They claimed that the yield of high-quality DNA preparations decreased when the study material was not stored at lower temperatures (Słomski et al. 2014).

Qualitative analysis was carried out by means of electrophoresis in agarose gel. Smears were apparent along the lanes in all 24 analyzed samples from the *Cervidae* bones. The gels did not feature high molecular mass DNA, which indicated that the genetic material was fragmented, i.e. it was present, but in small quantities.

There are numerous literature reports describing challenges of bone material analysis. Under perfect conditions, high molecular mass DNA (exceeding 50 kbp) should be visible in the form of a distinct band. DNA smears in the agarose gel suggest degradation of the nucleic acid (Słomski 2014).

It is worth noting that one of the major difficulties in working with the bone material is the activity of endogenous nucleases that catalyze hydrolytic breakdown of DNA and become active immediately after the death of an organism, causing gradual degradation of DNA (Alaeddini et al. 2010).

In the view of Słomski et al. (2008), the default DNA isolation methods from remains, including bone tissues that were exposed to various environmental conditions, are unsuitable for DNA isolation. The authors suggest that for the column-based method (most common DNA isolation method) guanidinium isothiocyanate (GTC) should be added to improve quality and efficiency of deoxyribonucleic acid isolation.

These researchers isolated DNA from aurochs bone. However, it was highly degraded, visible in the agarose gel as a distinct smear (Słomski et al. 2008). Based on size comparison of DNA isolates electrophoretically separated against the λ DNA/HindIII, EcoRI DNA ladder, genomic DNA in fragments of approx. 900 bp and 200-300 bp was obtained.

It seems that irrespective of the method used for DNA isolation from bone, most often the obtained DNA is degraded as a result of exposure to factors such as temperature or moisture; it also depends on the time and place of storage of the biological material. In summary, it may be concluded that there is no effective method enabling isolation of high molecular mass DNA from problematic materials such as bone tissues.

5. CONCLUSIONS

1) The highest DNA concentration was observed in sample K5 (1264.2 ng/µl).
2) In sample K10, the DNA isolate concentration was the lowest (12.9 ng/µl). This sample was also the most contaminated.
3) Isolation of DNA from bone material is both labor- and time-consuming.
4) The value of absorbance coefficient A260/A280 decreased with increasing weight of the bone powder.
5) The Bone DNA Purification Kit manufactured by Eurx may be effective in DNA isolation only from bones stored in good conditions.

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