DETERMINATION OF THE SUITABILITY OF AGILENT BIOANALYZER 2100 FOR INVESTIGATIONS INTO WILDLIFE CRIMES: CASE STUDIES

KRISTÝNA HEBENSTREITOVÁ[1,*], LENKA VAŇKOVÁ[1,2] AND DANIEL VANĚK[1,2,3,4]

[1] Institute for Environmental Studies, Charles University, Benátská 2, 128 01 Prague, Czech Republic
[2] Forensic DNA Service, Budínova 2, 180 81 Prague, Czech Republic
[3] Bulovka University Hospital, Budínova 2, 180 00 Prague, Czech Republic
[4] 2nd Faculty of Medicine, Charles University, Prague, Czech Republic
* Corresponding author: hebenstk@natur.cuni.cz

ABSTRACT

This article examines the use of chip electrophoresis in wildlife crime investigations through three mock case studies. Specifically, we analysed DNA extracted from the tanned hide of *Panthera pardus*, a species protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), at various stages of the tanning process (Case study #1). Tanned hides present a unique challenge due to the detrimental effects of tanning on DNA integrity, resulting in highly degraded DNA extracts. Therefore, assessing DNA integrity before performing standard DNA analyses is critical to conserving laboratory resources. One of the conventional methods for evaluating DNA integrity involves determining the degradation index using quantitative polymerase chain reaction (qPCR). This study explored whether chip electrophoresis, a faster and simpler alternative to qPCR, could provide comparable assessments of DNA integrity. In addition, its applicability for use in established assays, such as the Triplex assay, which is used for rapidly classifying unknown biological material (Case study #2) and species identification based on mitochondrial DNA lenght polymorphism (Case study #3). The findings indicate that while chip electrophoresis is effective in established assays, it is unsuitable for assessing the quality of DNA extracted from tanned hides.

Keywords: chip electrophoresis; DNA degradation; DNA quality control; lab-on-chip; wildlife forensics

Introduction

In recent years, forensic science has undergone a major change as it now uses modern technologies to increase the precision and efficiency of investigative processes.

In the early stages of an investigation, obtaining DNA analysis results quickly is critical for ensuring their relevance and utility in guiding the investigative process (Mapes et al. 2015). This is particularly significant in cases of wildlife crime, where the process of gathering evidence is often complex and challenging (Henger et al. 2023), especially as the DNA analysis can take several days to complete.

Forensic wildlife DNA analysis typically focuses on three key areas: species identification, individual identification, and determining relationships between individuals. The analysis typically involves several steps: collecting and preserving biological samples, extracting and amplifying DNA, sequencing or fragment analysis, and comparing it to reference databases for species or individual identifications. Despite its importance, there are significant challenges, including sample degradation, limited reference data, resource constraints and the need for the expertise of specialists (Kumar et al. 2017). These limitations need to be addressed if the accuracy of wildlife forensic DNA analysis is to be improved.

Devices that provide immediate information on a forensic sample are particularly valuable in facilitating rapid and effective analyses. "Lab-on-a-chip" (LOC) technology is a promising solution as it simplifies the analysis and reduces the time and resources required. A LOC is a device that integrates a laboratory technique within a small chip. Consequently, LOC devices provide swift analyses, require minimal quantities of analyte and are portable (Bruijns et al. 2016).

These new technologies are helping to overcome some of the difficulties in analysing a forensic sample, such as the low resolution of agar electrophoresis in multiplexed protocols or expansivity in the routinary use of capillary electrophoresis (CE). CE generally provides a better resolution than standard agarose gel electrophoresis, but is costly for routine use (Gupta et al. 2010).

The main focus of this article is to explore if it is possible to incorporate the Agilent Bioanalyzer 2100 in the workflow of established assays. We tested the Bioanalyzer in three different mock case studies: #1) Assessment of DNA quality in extracts from samples of a *Panthera pardus* hide in different stages of tanning; #2) Rapid classification of unknown biological material using Triplex assay; and #3) Species identification based on the length polymorphism of the mitochondrial DNA control region (CR-mtDNA).

Each of these studies involves a different method commonly used in forensic analyses (qPCR, Sanger sequencing, Fragment analysis). This article aims to determine whether the Agilent Bioanalyzer 2100 can be used for these analyses and provide similar results.

The first study aimed to assess the quality of the DNA extracted from the tanned hide of *Panthera pardus*. It is often challenging to obtain amplifiable DNA from

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tanned hides and furs as it is often degraded (Vuissoz et al. 2007; Hebenstreitova et al. 2024). This is further compounded by the presence of various chemicals and materials used during the tanning process, many of which are PCR inhibitors and also hinder the amplifiability of DNA extracted from tanned hides (Hebenstreitova et al. 2024). However, this is typical of biological samples collected in cases of wildlife crime (Hedmark and Ellegren 2005; Prakash Goyal 2016; Khan et al. 2018; Khedkar et al. 2019; Rajani et al. 2020). Numerous analyses used in wildlife forensic genetics, such as DNA typing or species identification using Sanger sequencing, depend on a certain quantity of amplifiable DNA (Zou et al. 2015). Given that these analyses are both costly and time-consuming, a tool providing a quick assessment of the quality of DNA before these analyses would be advantageous. The conventional approach for assessing DNA quality relies on determining the degradation index (DI) based on quantitative polymerase chain reaction (qPCR). However, the continuous search for faster and more accessible methodologies prompts the exploration of alternative techniques.

The Agilent Bioanalyzer 2100's sensitivity allows the determination of the level of DNA degradation purely on extracted DNA, thus overcoming possible bias due to the effect of inhibitors that affect qPCR.

The Triplex assay is used for the rapid identification of mammalian, nonmammalian and plant DNA in samples of unknown origin (Saskova et al. 2017). The Triplex assay protocol involves a PCR amplification targeting the rbcL gene in plants (RuBisCO), the COI gene in animals (cytochrome oxidase I), and the artificial internal positive control in a single reaction. Visualization of the PCR products via gel electrophoresis then provides information on the presence of animal or plant DNA in the sample. This step can be followed by Sanger sequencing and species identification. The visualization of PCR products can be done using chip electrophoresis instead of agarose gel electrophoresis. Similarly, chip electrophoresis can be used for the identification of species based on the length of the mitochondrial control region (CR-mtDNA) instead of capillary electrophoresis, which speeds up the laboratory process. This method is beneficial when dealing with a mixture of several species in one sample, but can also be used for species assignment in single-source samples (Pun et al. 2009; Vankova and Vanek 2022). However, important forensic validation is needed for the routine use of the Agilent Bioanalyzer 2100.

Materials and Methods

Case study #1

In this study, the hide of *Panthera pardus* that died in Jihlava ZOO was used for tanning. The tanning process was carried out by a professional, using three different commercially available tanning agents: Lutan FN (L, aluminum sulfate), Novaltan AL (N, aluminum triformate) and a chrome-based agent (C, chromium sulfate), all sourced from Bauer Handels, Switzerland. The process (Fig. 1) begins with pre-tanning procedures, which involve trimming the underside of the hide to produce smooth leather (fleshing) and removing impurities, degreasing and breaking down globular proteins (soaking). A two-stage tanning process follows, preceded by acidification (pickling) to enhance the penetration of tanning agents and further thinning. The finishing steps ensure the leather is lubricated (fat liquoring), soft and dry, which improves its durability and resistance (Pachnerová Brabcová et al. 2024).

The hide was labelled at the different stages of the tanning process, as detailed in Fig. 1. Comprehensive details of each tanning stage are described by Hebenstreitova et al. (2024).

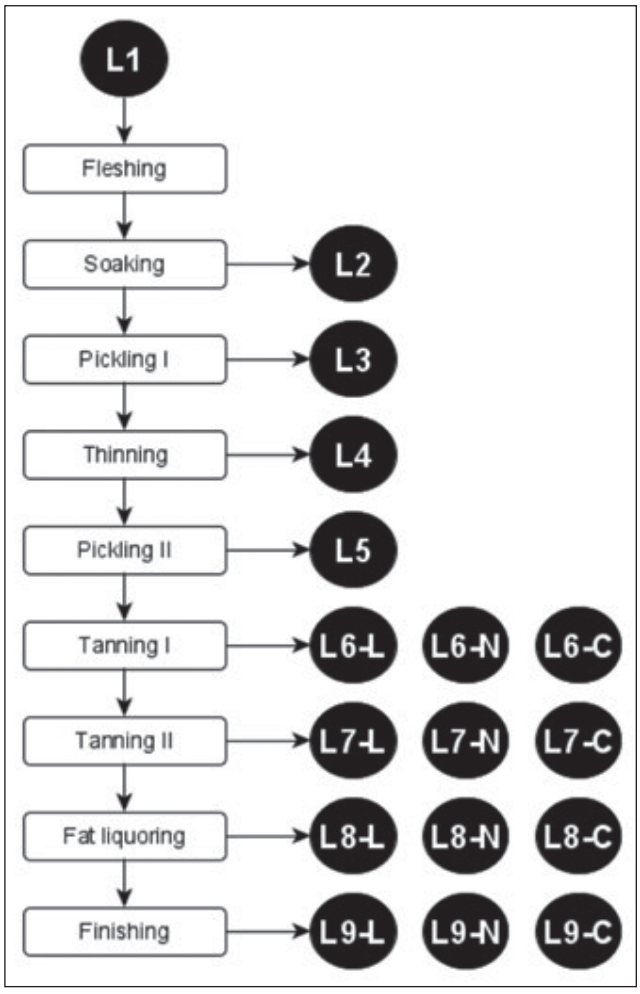

Fig. 1 The tanning process consisted of nine steps (indicated by rectangles), with samples collected at specific stages (dark circles). The first sample (L1) was collected before the tanning process began. After the fifth step, the process differed depending on which of the three tanning agents was used, as indicated in the sample labels: 'L' for Lutan, 'N' for Novaltan, and 'C' for chromium sulphate (as published by Pachnerová Brabcová et al. 2024).

An optimized protocol for extracting genomic DNA (gDNA) from tanned hides was used, which utilized the commercially available Quick-DNA Miniprep Plus Kit (Zymo Research, USA). A detailed description of this

protocol was previously published by Hebenstreitova et al. (2024).

Before analysis using the Agilent Bioanalyzer 2100, all of the DNA extracts were quantified by a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). DNA isolates exceeding 500 pg/µl gDNA were diluted to a final DNA concentration of 250 pg/µl gDNA, which is compatible with the quantitative range of the high-sensitivity DNA kit (Agilent Technologies, USA).

Case study #2

For this study, DNA was extracted from the muscle tissue of *Bos taurus* using the Quick DNA Miniprep kit (Zymo Research) and from the leaves of *Hibiscus* sp. using Quick DNA Plant/Seed Miniprep kit (Zymo Research) according to the manufacturer instructions. The Triplex assay was done according to a previously published protocol (Saskova et al. 2017) with minor changes to the PCR reaction. Five µl of gDNA was amplified (concentration range from 2–4 ng/µl) in a final volume of 25 µl containing 2.5 µl of 10x PCR Buffer II (Thermo Fisher Scientific), 2 µl of 25 mM $MgCl_2$ (Thermo Fisher Scientific), 0.5 µl of 10 mM dNTPs, 1 U of AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific), 1 µl of each 10 µl primer and DNase/RNase-Free Water were added to the final volume. The DNAs of *Bos taurus* and *Hibiscus* sp. were used as template DNA.

Before analysis on the Agilent Bioanalyzer 2100, all of the PCR products were quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). Products exceeding 500 pg/µl gDNA were appropriately diluted to achieve a final DNA concentration of 250 pg/µl gDNA, which ensures compatibility with the quantitative range of the high-sensitivity DNA kit (Agilent Technologies, USA).

Case study #3

For this study, DNA was extracted from various tissues (blood, faeces, muscle tissue and hair) from seven different species of mammal (*P. leo*, *P. tigris*, *P. pardus*, *B. taurus*, *S. scrofa domesticus*, *O. cuniculus* and *G. gallus*) using the Quick-DNA Miniprep Plus Kit (Zymo Research) according to the manufacturer's instructions. All of these tissue samples were provided by various Zoos in the Czech Republic.

The hypervariable domain of the mitochondrial control region (CR-mtDNA) was amplified using previously published primers and protocols (Pun et al. 2009; Vankova and Vanek 2024). The only variation was that unlabelled primers were used in this case.

Before analysis using the Agilent Bioanalyzer 2100, all of the PCR products were quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). Products exceeding 500 pg/µl gDNA were appropriately diluted to achieve a final DNA concentration of 250 pg/µl gDNA, which is compatible with the quantitative range of the high-sensitivity DNA kit (Agilent Technologies, USA).

Agilent Bioanalyzer 2100

This analyser was used to visualize and determine the quality of the DNA. It is a microfluidics-based electrophoresis instrument that allows for separation and quantitation of DNA down to pg/µl sensitivity. It is well-established and commonly used for DNA library quality control in next-generation sequencing (Agilent Bioanalyzer System 2018).

The samples were analysed using a high-sensitivity DNA kit according to the protocol provided by the manufacturer. The Agilent High Sensitivity DNA Kit is designed for determining the size and quantity of fragmented DNA in DNA sequencing libraries and DNA samples derived from ChIP. This tool has a 45-minute runtime and a size range of 35 to 10 380 bp and can accommodate 11 samples per chip. It provides a size resolution of ±10% for 50–600 bp and ±20% for 600–7000 bp, with a sample volume requirement of 1 µl. This kit, which remains stable for four months, includes 10 chips (110 samples total) and has a size accuracy of ±10%, size precision with a 5% coefficient of variation (CV), quantitative accuracy of 20%, and quantitative precision of 15% CV for 50–2000 bp and 10% CV for 2000–7000 bp, within a quantitative range of 5–500 pg/µl. The maximum buffer concentration allowed is 10 mM Tris and 1mM EDTA (Panaro et al. 2000; Agilent Technologies 2009). The assay validation followed the ANSI/ASB standard for the internal validation of forensic DNA analysis, as partly described by Webster et al. (2023).

Sensitivity: The analytical sensitivity corresponds to the quantitative range indicated by the manufacturer, which is 5–500 pg of DNA.

Robustness: Robustness was tested using an Agilent Bioanalyzer 2100 in three different assays: quality control of tanned hide DNA extracts, Triplex assay and CR-mtDNA typing, as described above.

Repeatability: Amplicons resulting from the CR-mtDNA and Triplex assays were used to test the repeatability.

Reproducibility: This was tested by running two assays independently.

Specificity: This approach is universal and is used for nucleic acid separation.

Quality control: Negative and positive controls were run in all of the above experiments.

Results

Case study #1

In this study, 17 electropherogram plots were obtained. In samples taken during the preparatory stages of leather-making (L1–L5), fragments of > 1000 bp were recorded (Fig. 2). From the onset of the leather-making process, there is a noticeable shift towards shorter fragments, with fragments ≥ 1000 bp virtually absent (Figs 3–5). In addition, the DNA in leather samples treated with chromium sulphate was the most degraded, as no

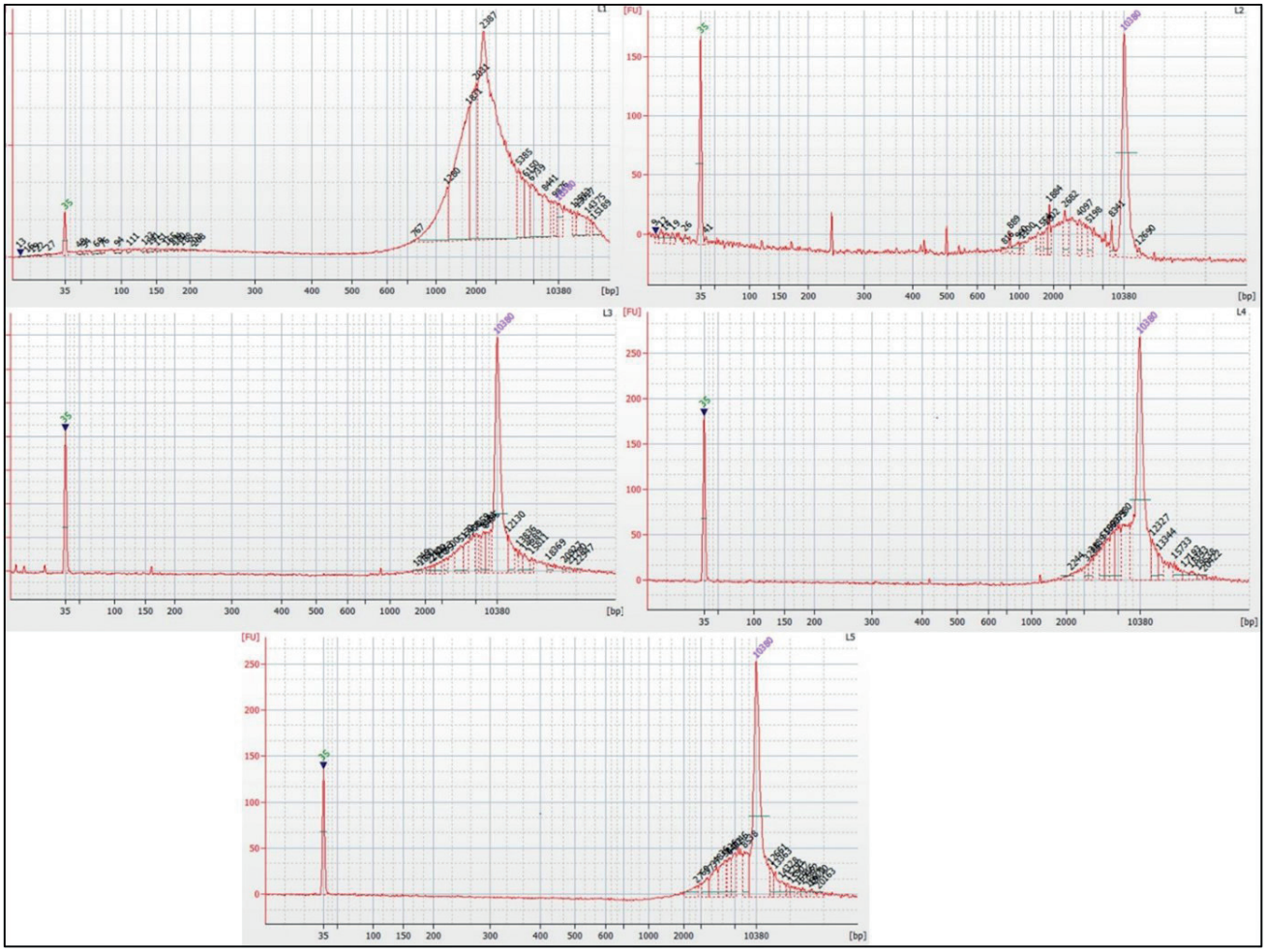

Fig. 2 Electropherograms recorded for samples L1 to L5, respectively, using the Agilent Bioanalyzer 2100. The values on the y-axis are units of fluorescence (FU) and on the x-axis fragment lengths in base pairs (bp). The size range is defined by two distinct peaks: the position of the lower is for 35 bp and the upper for 10,380 bp.

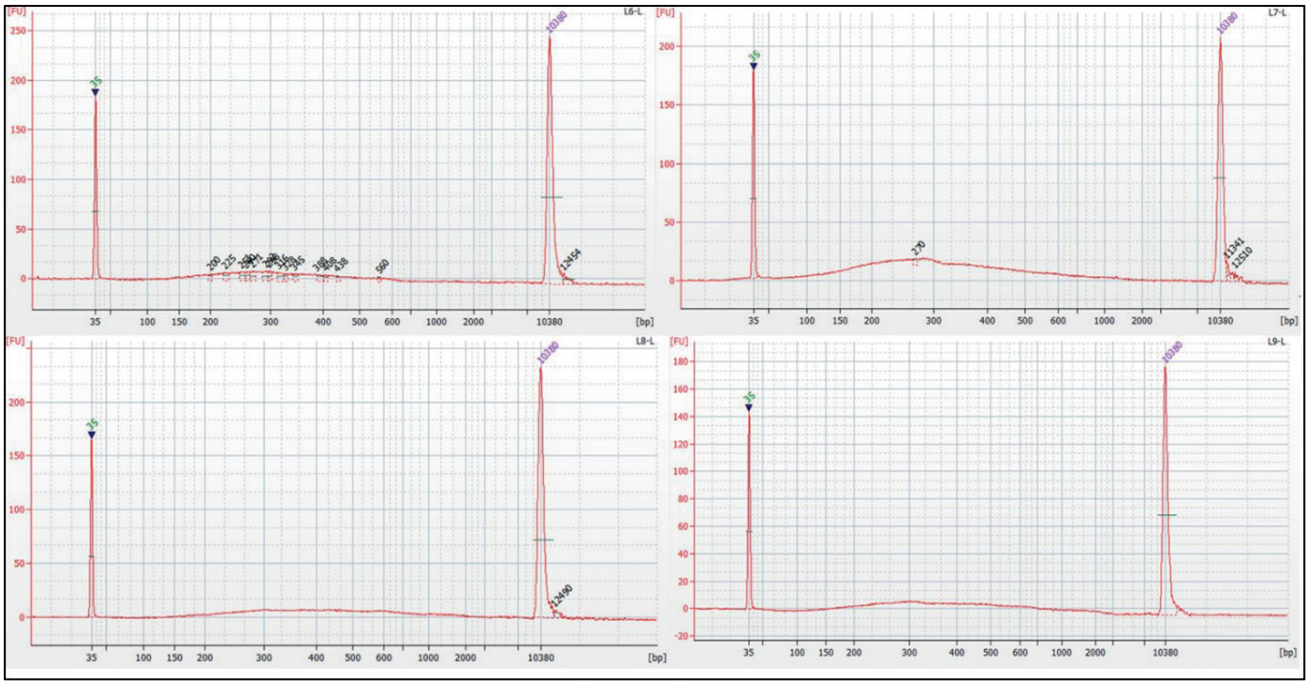

Fig. 3 Electropherograms recorded for the Lutan FN-tanned samples L6 to L9, respectively, quantified using the Agilent Bioanalyzer 2100. The values on the y-axis are units of fluorescence (FU) and x-axis fragment lengths in base pairs (bp). The size range is defined by the positions of two distinct peaks: the first at 35 bp and the second at 10,380 bp.

DNA fragments were detected from stage L6 onwards (Fig. 5). A lower level of DNA fragmentation was recorded in leather from L6 onwards treated with Lutan (Fig. 3) and Novaltan (Fig. 4).

For samples L1 to L5, there are fragments larger than this method's lower and upper range (35 bp to 10,380 bp) (Fig. 2). However, the concentration of fragments beyond the detection range cannot be determined. In sample L1, the largest are fragments ranging from 15,000 bp to 9,000 bp. For samples L2 to L5, the number of large fragments decreases, although they are still present, with the distribution of all the recorded fragments being roughly comparable (Fig. 2).

For the samples L6 to L9, it was not possible to determine the distribution of individual fragments (Figs 3–5). Sample L6 contained fragments ranging in size from

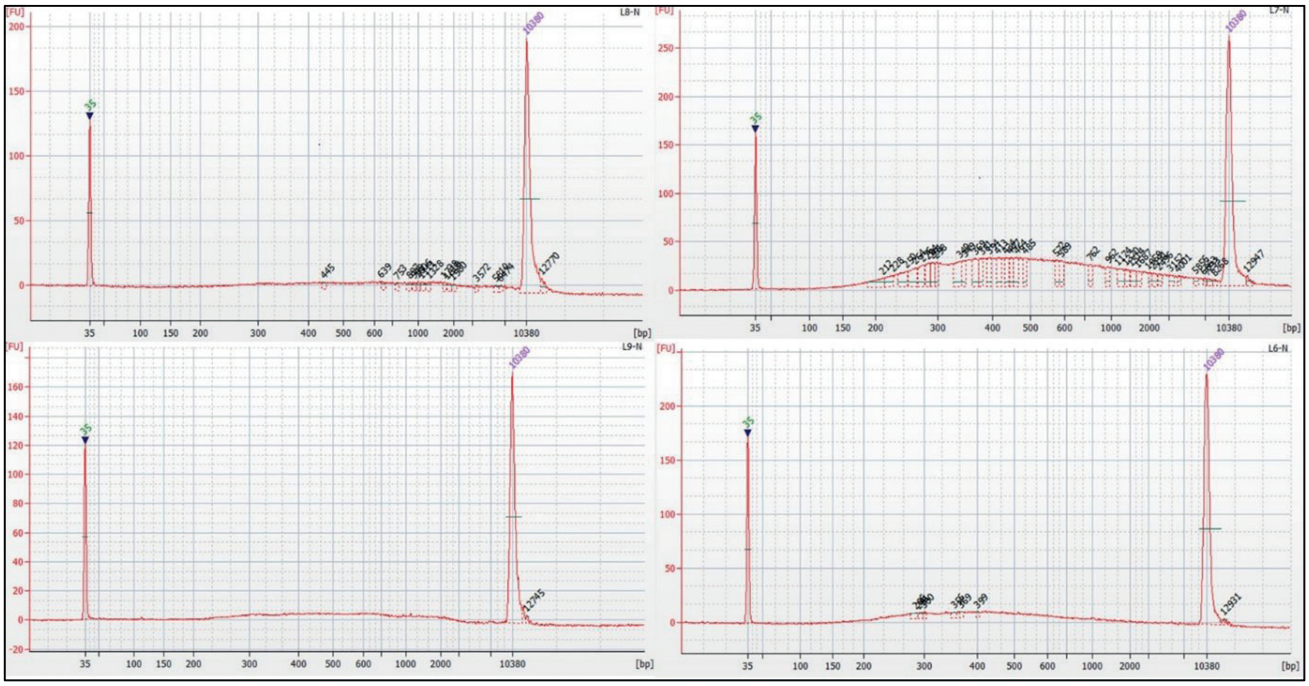

Fig. 4 Electropherograms recorded for the Novaltan AL-tanned samples, L6 to L9, respectively, quantified using the Agilent Bioanalyzer 2100. The values on the y-axis are units of fluorescence (FU) and the x-axis fragment lengths in base pairs (bp). The size range is defined by two distinct peaks: the first is for 35 bp and the second for 10,380 bp.

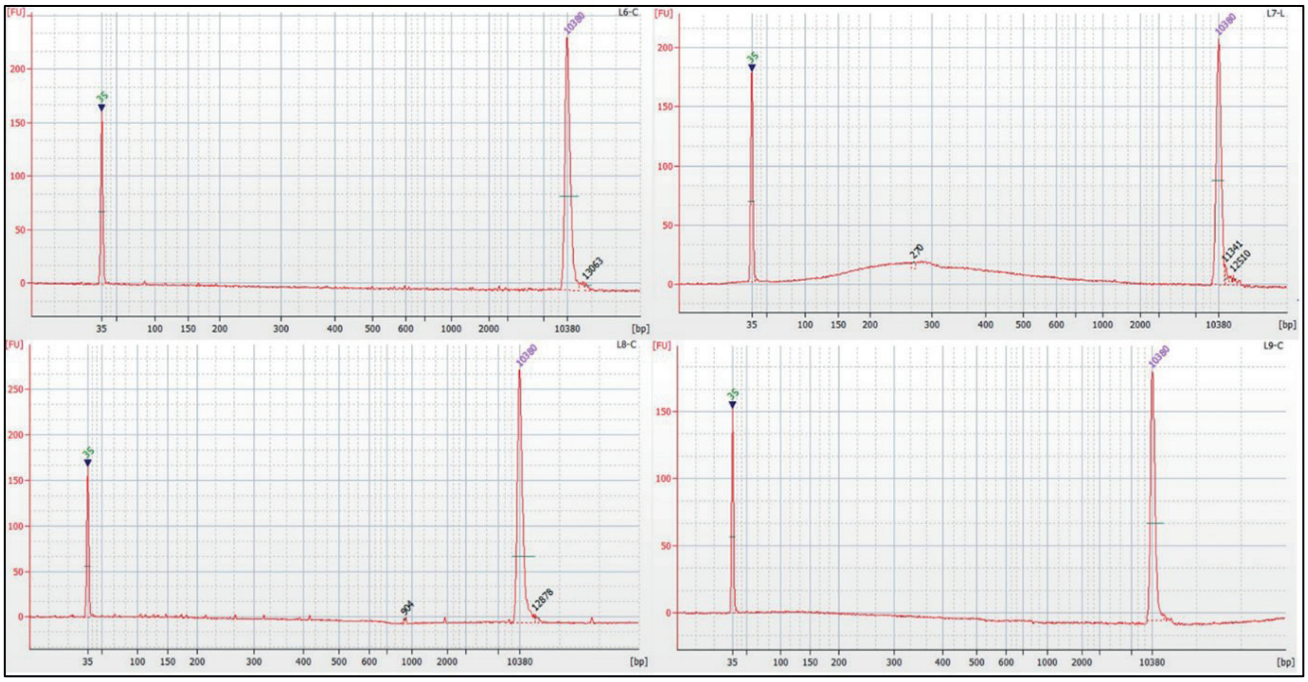

Fig. 5 Electropherograms recorded for the chromium sulphate-tanned samples, L6 to L9, respectively, quantified using the Agilent Bioanalyzer 2100. The values on the y-axis are units of fluorescence (FU) and x-axis fragment lengths in base pairs (bp). The size range is defined by the positions of the two distinct peaks: the first is for 35 bp and the second 10,380 bp.

approximately 200 bp to 500 bp (Fig. 3). Sample L6-N contained fragments ranging in size from 300 bp to 400 bp (Fig. 4). The presence of larger fragments of approximately 445 to 7,000 bp, was unexpectedly recorded in sample L8-N (Fig. 4), but is consistent with the DNA quantification reported using other methods (Hebenstreitova et al. 2024).

Case study #2

For this study, three electropherograms were recorded and are similar to those reported by Saskova et al. 2017 (Fig. 6). In the sample containing the DNA of *Bos taurus* (Fig. 5B), a distinct peak at approximately 525 bp was recorded, indicating the presence of animal DNA in that sample. In the sample containing *Hibiscus* sp. DNA

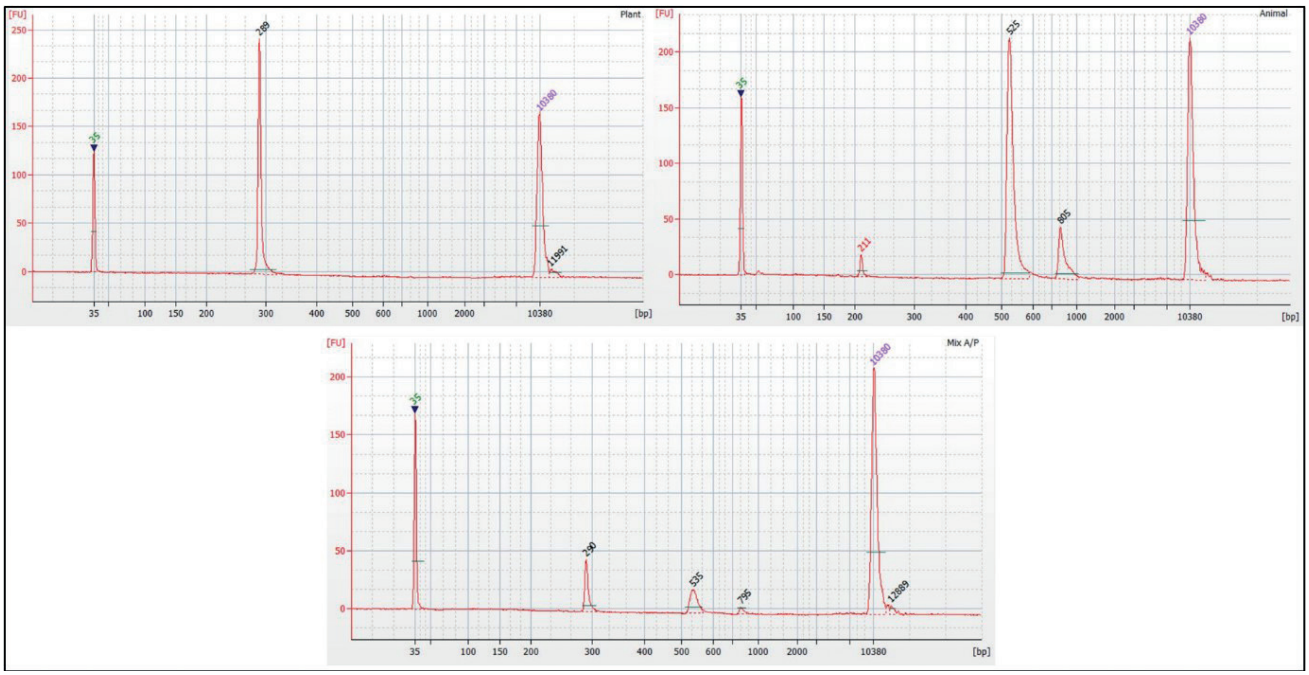

Fig. 6 Electropherograms recorded for the Triplex assay samples visualized using the Agilent Bioanalyzer 2100. (A) The sample containing *Hibiscus* sp. DNA is indicated by the peak at 289 bp. (B) Sample containing *Bos taurus* DNA is indicated by the peak at 525 bp. (C) Sample containing a mixture of plant and animal DNA (*Hibiscus* sp. and *Bos taurus*) is indicated by the peaks at 290 bp and 535 bp. The values on the y-axis are units of fluorescence (FU) and the x-axis fragment length in base pairs (bp). The size range is defined by two distinct peaks: the first at 35 bp and the second at 10,380 bp.

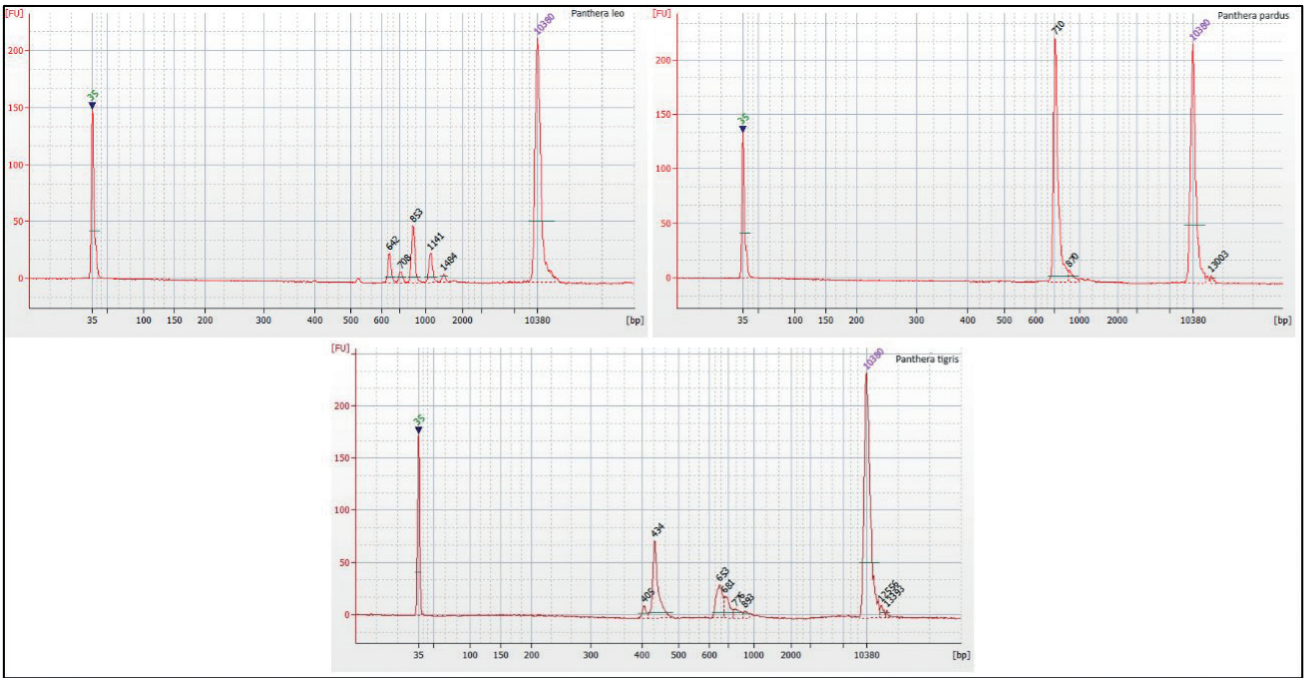

Fig. 7 Electropherograms recorded for the CR-mtDNA assay samples from *P. leo*, *P. pardus*, and *P. tigris*, respectively, visualized using the Agilent Bioanalyzer 2100. The values on the y-axis are units of fluorescence (FU) and the x-axis fragment lengths in base pairs (bp). The size range is defined by the positions of two distinct peaks: the first at 35 bp and second at 10,380 bp.

(Fig. 5A), there was a distinct peak at approximately 289 bp, indicating the presence of plant DNA. In the sample containing a mixture of animal and plant DNA (Fig 5C), two peaks were recorded at 535 bp and 290 bp, respectively, indicating the presence of animal and plant DNA. The amplification of the internal positive control (IPC) is marked by the peak at approximately 805 bp and 795 bp.

Case study #3

For this study 7 electropherogram plots were obtained (Fig. 7 and Fig. 8), which indicate that the length of the amplified control region of mtDNA in in different animals (*P. leo*, *P. tigris*, *P. pardus*, *B. taurus*, *S. scrofa domesticus*, *O. cuniculus* and *G. gallus*) is variable.

Discussion

The Agilent Bioanalyzer 2100 is a very useful tool for molecular biology and genetics, with advantages over traditional methods for determining the size and quantity of DNA, such as, agarose gel electrophoresis, spectrophotometry and fluorometry. Unlike agarose gel electrophoresis, which has limited resolution and requires larger sample volumes, the Bioanalyzer provides high-resolution separation of DNA fragments with increased sensitivity (Vitale 2000; Lu et al. 2002). Compared with PAGE, the Bioanalyzer eliminates the need for labour-intensive gel casting and staining (Agilent Technologies 2009). The Bioanalyzer's quantitative range (5–500 pg of DNA) enables the detection and separation of the minute amounts of DNA a characteristic of forensic samples, which make it highly sensitive.

The Bioanalyzer's microfluidic chip-based technology minimizes the size of the samples needed and in so doing preserves the valuable evidence. Furthermore, its automated nature reduces analysis-to-analysis variability and minimizes the risk of contamination, ensuring high assay reproducibility and repeatability.

In this study, the suitability of the Bioanalyzer for wildlife forensic genetics was explored. The results indicate that while the Bioanalyzer may not be particularly suitable for quantifying nonamplified DNA, it can be used for assessing the quality of DNA extracts. Another method of assessing DNA quality is to determine the degradation index using qPCR (Vernarecci et al. 2015) or a visual assessment using gel electrophoresis (Bhoyar et al. 2024). The main limitations of using qPCR to determine degradation is the need for the optimization of reaction conditions, the presence of PCR inhibitors and tendency to overstate the concentration of short DNA fragments (Smith and Osborn 2009; Gill et al. 2022). Agarose gel electrophoresis can provide insight into the level of DNA degradation by comparing the pattern of fragment migration of degraded and non-degraded DNA, which results in a typical smear pattern (Mohamed et al. 2020; Bhoyar et al. 2024).

The Agilent Bioanalyzer 2100 is used in forensic, archaeological, and ancient DNA studies, particularly for quality control and analysis of DNA samples (Senst et al. 2024). In forensic studies, it is used for evaluating the quality of degraded postmortem DNA samples, assessing total DNA and adapter dimer concentrations essential for next-generation sequencing (NGS) library preparation and quantifying mitochondrial DNA in order to refine inputs for cycle sequencing (Lozano-Peral et al.

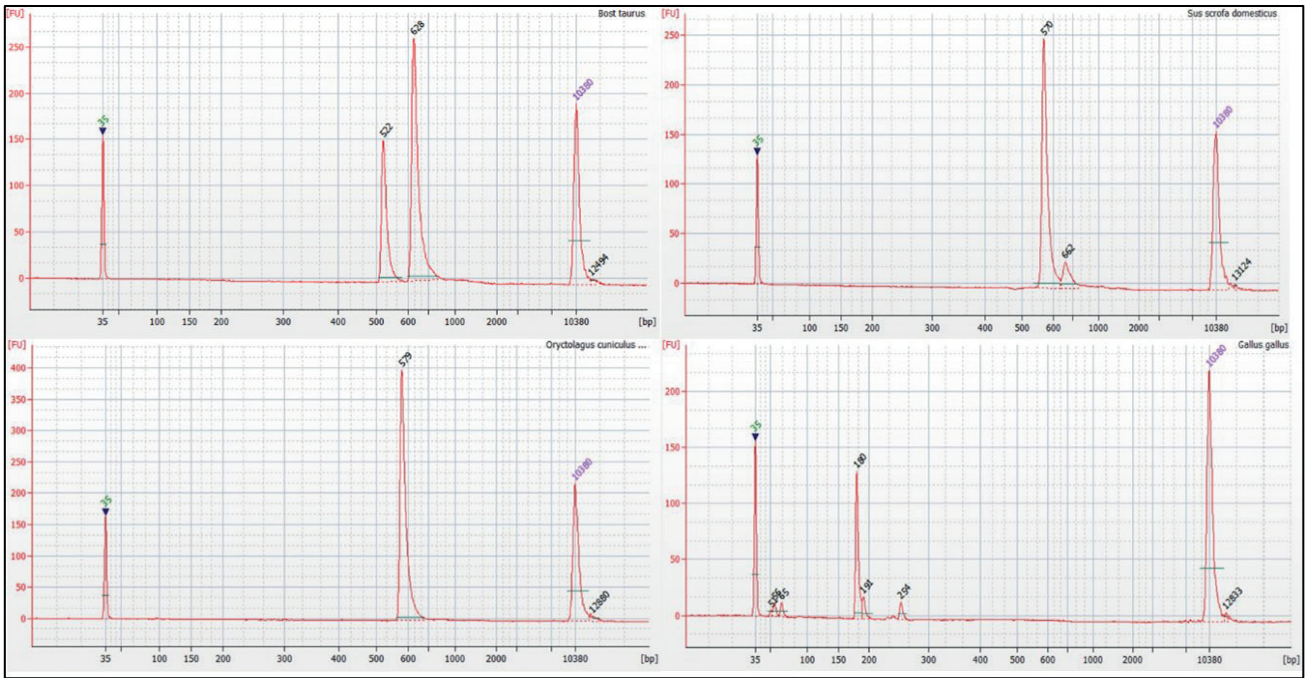

Fig. 8 Electropherograms recorded for the CR-mtDNA-assay samples from *B. taurus*, *S. scrofa domesticus*, *O. cuniculu*, and *G. gallus*, respectively, visualized using the Agilent Bioanalyzer 2100. The units on the y-axis are units of fluorescence (FU) and the x-axis fragment lengths in base pairs (bp). The size range is defined by two distinct peaks: the first at 35 bp and the second at 10,380 bp.

2021; Office of Chief Medical Examiner, n.d.; Senst et al. 2024). Archaeological and ancient DNA research has also benefited from its use, for instance, in the analysis DNA integrity under thermal stress and for improving extraction techniques for ancient samples under ultra-clean conditions (Matsvay et al. 2019). The examples demonstrate the instrument's versatility in ensuring analytical precision and advancing research in different scientific domains.

Using the "bioanalyzer" to evaluate the degradation of DNA extracts from tanned *Panthera pardus* hides at various stages of tanning proved useful for rapidly assessing the quality of the DNA extracted. This assessment is crucial, as it aids in determining whether a sample is of sufficient quality for costly and time-consuming methods such as DNA sequencing or typing, with a minimal use of the sample (1 µl/sample). The Bioanalyzer provided insights into the sizes and distribution of DNA fragments within an extract, and these results are consistent with previous findings, i.e., that electropherograms indicate that the leather-making process increases DNA fragmentation (Figs 2–5) (Hebenstreitova et al. 2024).

In addition to analysing nonamplified DNA, the Bioanalyzer can be utilized in assays involving PCR. The application of this approach for classifying unknown biological material via a Triplex assay and species identification via CR-mtDNA analysis was determined. The details of both these assays was previously published by Saskova et al. (2017). The resulting electropherograms were consistent with expectations based on previous findings, indicating that the Bioanalyzer can be integrated into PCR-based assays, however, neither classical agarose gel electrophoresis nor chip electrophoresis has the resolution of CE (Weiss et al. 1995; Gupta et al. 2010).

In species identification based on the analysis of the CR-mtDNA region, the results correspond with those previously published by Vankova and Vanek (2024), Saskova et al. (2017) and Pun et al. (2009) (Fig. 7 and Fig. 8), confirming the suitability of chip electrophoresis for species identification. The only variation from previously published results was recorded for the *Bos taurus* sample. According to Pun et al. (2009), there should be only one peak (537–544 bp); however, there are two distinct peaks. This could be attributed to nuclear-mitochondrial segments (NUMTs) (Zhang and Hewitt 1996; Song et al. 2008).

In addition to the aforementioned assays, the Agilent Bioanalyzer 2100 can theoretically be utilized in various assays, even beyond its intended scope of application. For example, it can be used in the analysis of polymorphic markers for identification purposes, such as variable number tandem repeats (VNTRs) or short tandem repeats (STRs) (Aboud et al. 2015); tissue or body fluid identification using mRNA analysis (Lin et al. 2015; Sauer et al. 2017); and field-based identification of illegal drugs (Lloyd et al. 2011). Another possible use is to include the Bioanalyzer in the species barcoding for the quality control of the mtDNA amplification prior to Sanger sequencing (Zakharov et al. 2011; Blekhman et al. 2020; Baur et al. 2022). The current exploratory application of the Agilent Bioanalyzer 2100 demonstrated that although it is unlikely to replace the established methods, this instrument can contribute to forensic investigations and research in specific cases. However, if the instrument is to be deployed in forensic analysis, further testing and validation for forensic use is required. These findings are consistent with those reported by other researchers (Gorzkiewicz et al. 2010).

Conclusion

In conclusion, this method can be used for carrying out various assays in wildlife forensic investigations or research. While it is not particularly suitable for the quantification of DNA in extracts from tanned skins, as the results for stage 6 and onwards in the tanning process are only of limited value, it can be particularly useful in the quick assessment of the quality of DNA extract before costly and time-consuming methods such as DNA sequencing or DNA typing. However, this method is suitable for rapid sample classification and identification of species.

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Data availability statement: Data supporting this study's findings are deposited on Zenodo.org with the accession link: https://doi.org/10.5281/zenodo.13777822.

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