Evaluating non-destructive sampling methods of parchment for genomic sequencing

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Parchment is a writing surface derived from animal skins. While sequencing the mitochondrial genome has been used to identify the parchment animal source, non-destructive sampling methods are pivotal because of their unique and irreplaceable nature. In this study, four different parchments were utilized to evaluate three non-destructive sampling methods: brushing, gecko tape, and forensic fibre lifts. For all methods, the impact of a 30-second eraser pre-cleaning to remove surface contaminants was assessed. After sample collection, total DNA was isolated, the mitochondrial genome enriched and sequenced using Illumina chemistry, and sequencing reads processed through a bioinformatics pipeline. Across all four documents, brushing with an eraser pre-cleaning was the optimal method, with an average of 98% of the mitochondrial genome recovered. Regardless of sampling method, collection after a 30-second eraser pre-cleaning resulted in higher source species DNA sequences. Non-destructive sampling will preserve documents with historical significance while allowing for genetic analysis.

Primarily used in medieval times (500–1500 AD), parchment is a writing surface derived from animal skins[1-4]. The animals most commonly used to make parchment include cattle (*Bos taurus*), sheep (*Ovis aries*), and goat (*Capra hircus*)[5-15]. The laborious parchment-making process involves soaking the animal skin in a water and lime bath to allow for loosening of the hair fibers. Following this, the skin is cleaned with a sharpened tool until all hair and excess skin is removed[1]. After being stretched and dried, the skin is ready to be used as a writing surface. Parchment was often used to document information of importance, such as financial transactions, legal records, and religious writings. Given parchment is an animal product, it also contains valuable genetic information from the animal source and any biological product deposited on the surface[16]. The use of modern molecular approaches to analyze historical deoxyribonucleic acid (DNA) contained within, or on the surface of, parchment has the potential to provide previously unattainable insight into medieval societies and practices. Conventional methods to collect biological material for DNA analysis are inherently destructive and consumptive (e.g., cutting, dissecting) and so are not suitable for parchment documents which hold cultural and historical significance.

To harness the value of parchment as a reservoir of biological information, researchers have explored various non-destructive methods to collect material for scientific analysis[9,10,12,17-21]. Interactions between parchment and light under varying wavelengths (ultraviolet and near infrared) as measured by a spectrophotometer, have been used to determine parchment source species[17]. The utility of PVC eraser crumbs/rubbings – a byproduct of a cleaning method commonly used by conservators maintaining parchment – was assessed by Fiddyment and colleagues[18] who collected cellular material from 72 pocket bibles made of ultrathin parchment. The subsequent eraser crumbs were successfully subjected to peptide analysis (also known as proteomics) to determine parchment source species and it was determined that the source species was limited to the expected calf, goat and sheep traditionally used[18]. The PVC eraser has been classified in previous research as non-destructive collection method that does not require any special equipment or storage, allowing researchers or conservators to collect a sample at their convenience, without having to transport the artifact[18,19].

DNA-based analysis of non-destructively collected cellular material can also be used to identify source species through the analysis of the underlying DNA sequence[8,12,16,19,22,23]. Researchers have previously compared the recovery of source species cellular material (and by proxy DNA) between two non-destructive sampling methods – cytology brushing and PVC eraser rubbing – to destructive sampling (cutting) of parchment[3]. Scheible and colleagues[3] subjected isolated DNA collected using these three methods to hybridization capture to enrich mitochondrial DNA sequences – which are routinely used for DNA-based species identification – prior to Illumina sequencing[3]. Results showed that full/near full mitochondrial

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genome sequences were obtained for ~56% of non-destructively sampled parchments[3]. While this work highlighted that non-destructive sampling coupled with DNA sequencing can be used to successfully determine the animal source of the parchment, incidental sequencing of surface contaminants of parchment (e.g., human DNA from repeated touching, animal-based surface treatments) can take up valuable space on the sequencing run. To maximize sequencing reads derived from the source animal, it is necessary to find non-destructive sampling methods that reduce the collection of surface contaminants. The present study aimed to evaluate three non-destructive sampling methods for the collection of cellular material from the parchment source species, both with and without an eraser pre-cleaning.

Methods

Parchment sampling

Parchments with known source species were selected from a previous study (Table 1)[3]. Three distinct non-destructive collection methods were utilized for sampling: cytology brushing[3], gecko tape, and forensic fiber lift. Each method was also tested with and without a PVC eraser pre-cleaning before sample collection, with the intent of assessing whether surface contaminants could be removed to permit an increase in the proportion of authentic parchment cellular material in the sample. Areas containing text or an accumulation of debris or surface contamination were avoided, including the gutter and the corners likely containing human cells from repeated touching (see Fig. 1). Mars Plastic Erasers (Staedtler, Nuremberg, Germany) were used to pre-clean the surface of the parchment (~30 s). To limit variability a larger area was marked in pencil and cleaned via eraser rubbing, prior to marking the smaller-sized areas for collection via one of the three methods (Fig. 1). All eraser crumbs generated during the pre-cleaning were removed before sample collection and were not subjected to downstream processing. Each document had two larger areas (~2.5 cm by 10 cm each) and eight smaller areas (~1.5 cm by 2.5 cm each) marked in pencil for sampling (see Fig. 1 for details).

Collection via each of the three methods was undertaken as follows. (1) *Cytology brushing*: EndoCervex-Brushes (Rovers, Lekstraat, Netherlands) were used to collect cellular material by carefully brushing the surface of the parchment (2.5 cm by 10 cm area) for one minute, turning the brush as needed to utilize all bristles. This process was then repeated in a second area (2.5 cm by 10 cm area) that had been subject to a 30 s eraser pre-cleaning. Following the collection via brushing, the tip of the brush was detached and placed in an appropriately labeled 1.7 mL LoBind tube (Eppendorf, Hamburg, Germany). (2) *Gecko tape*: A 1 cm by 2 cm piece of gecko tape, also known as nano grip tape (Jsiton, China), was cut using sterile tweezers and scissors to avoid external contamination. This tape is adhesive due to its nanostructures, which mimic that of a gecko's toes, rather than through chemical adhesives. Using tweezers, the tape was placed on the surface of the parchment (1.5 cm by 2.5 cm area) and pressed on lightly by the handles of the scissors. The tape was lifted off the parchment and placed on the same area again (for a total of two lifts). The gecko tape was carefully placed in an appropriately labeled 1.7 mL LoBind tube. A second piece of gecko tape (1 cm by 2 cm) was placed on a second 1.5 cm by 2.5 cm area of the parchment as described above, but for a total of five lifts. This process was then repeated (both two lift and five lift sampling) in additional 1.5 cm by 2.5 cm areas subjected to a 30 s eraser pre-cleaning. (3) *Forensic fiber lift*:

Fiber lift tape (SceneSafe, Essex, United Kingdom) was used to collect cellular material by cutting a 1 cm by 2 cm piece of tape using tweezers and scissors. The tweezers were used to place the tape on the surface of the parchment (1.5 cm by 2.5 cm area) and the handles of the scissors were used to lightly press down on the tape. The tape was lifted off the parchment and placed on the same area again (for a total of two lifts). The fiber lift tape was carefully placed in an appropriately labeled 1.7 mL LoBind tube. A second piece of fiber lift tape (1 cm by 2 cm) was placed on a second 1.5 cm by 2.5 cm area of the parchment as described above, but for a total of five lifts. This process was then repeated (both two lift and five lift sampling) in additional 1.5 cm by 2.5 cm areas subjected to a 30 s eraser pre-cleaning.

All sampling occurred in a sterile PCR hood, which was cleaned in between each parchment document with 10% bleach and treated with 15 min of ultraviolet light. Nitrile gloves were worn during the entire sampling process and new gloves were worn when handling a new parchment document. The tweezers and scissors were sterilized with a 10% bleach solution and allowed to dry before sampling and in between each parchment document. A total of 10 samples per parchment document were collected and sampling of one document using all methods was completed before moving onto the next. To assess possible contamination from sampling materials or the collection process, four sampling blanks were collected: (1) a clean EndoCervex-Brush placed straight into a clean, labeled 1.7 mL LoBind tube, (2) an unused 1 cm by 2 cm piece of gecko tape placed directly into a clean, labeled 1.7 mL LoBind tube, (3) an unused 1 cm by 2 cm piece of forensic fiber lift tape placed straight into a clean, labeled 1.7 mL LoBind tube, and (4) eraser crumbs generated from a clean eraser rubbed against a clean sheet of paper, carefully placed into a clean, labeled 1.7 mL LoBind tube. The eraser crumbs were collected into the tube using a single-use disposable spatula (VWR International, Radnor, PA). All samples and sample blanks (n, 44) were stored at room temperature when not in use.

DNA isolation

Isolation of DNA from collected samples was completed in four batches of 16 or fewer samples using a previously published method[20,24]. For every batch, a DNA isolation reagent blank was also processed to assess for any possible laboratory contamination. DNA isolations were completed as outlined by Scheible and colleagues[3] with the following modifications: (a) 750 μL of extraction buffer was added to each sample and reagent blank tube, and (b) a total of 31 μL was added to each MinElute Spin Column (Qiagen, Hilden, Germany) before the final elution spin. Isolated DNA was stored at −20 °C when not in use.

Library construction, hybridization capture and sequencing

Using the KAPA Hyper Prep Kit (Roche, Basel, Switzerland) libraries were constructed for each sample as described in Scheible and colleagues[3]. The following modifications were made: (a) half reactions were prepared using 25 μL of isolated DNA from samples or reagent blanks and Buffer EB (Qiagen) for the library negative, (b) KAPA unique dual-indexed adapters manufactured by Roche were used to individually barcode (also known as indexing) each sample for sequencing, (c) KAPA Pure Beads (Roche) were used for all bead purifications, and (d) purified libraries were quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, United States). A single equimolar pool of all 49 libraries (40 samples, four sampling and four DNA isolation blanks, and one library negative) was

Table 1 | Parchments selected for testing three non-destructive sampling methods

Parchment ID	Date of copying/manufacture	Text	Place copied	Source species
15-311	1472	Excerpt from I Kings, Chapter 8	Central France	Cattle (*Bos taurus*)
1785	1785	Indenture/release of dwelling	Devon, England	Sheep (*Ovis aries*)
1894	1894	Indenture/mortgage	Suffolk, England	Sheep (*Ovis aries*)
Modern	2012	N/A	N/A	Cattle (*Bos taurus*)

The following parchments were selected from T. Stinson's personal collection for sampling. The source species was determined by a previous study[3].
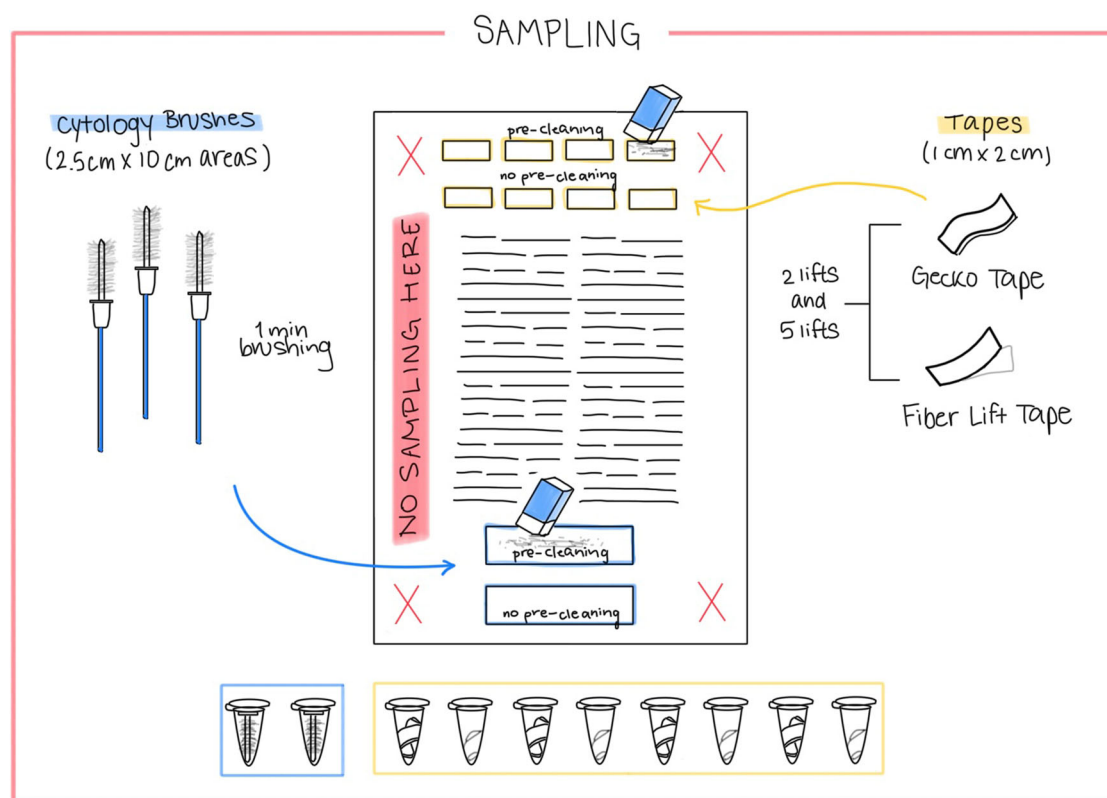
Fig. 1 | Strategy for non-destructive sampling of parchment across four documents. Cytology brushes were used for the collection of cellular material from an area ~2.5 cm by 10 cm ("larger areas") both with and without an eraser pre-cleaning. Additionally, two types of tapes were evaluated: gecko tape and forensic fiber lift. Samples were collected from an area ~1.5 cm by 2.5 cm ("smaller areas") with both tapes utilizing two lifts and five lifts. Tape samples were also collected both with and without an eraser pre-cleaning. Non-text and areas not highly touched were targeted for sampling.

prepared, with a maximum volume of 10 µL of any library added to the pool. Mitochondrial genome enrichment was achieved by hybridization capture, which has been shown to be compatible with challenging samples, notably those that contain highly fragmented DNA (~50–70 bp) or have been treated with chemicals[22,25–28]. Illumina sequencing was performed as specified in Scheible and colleagues[3].

Data analysis
Raw sequence reads were imported into the CLC Genomics Workbench (Qiagen) and processed through a published workflow[3], with the exception of both merged and non-merged reads being utilized in this workflow. JMP Pro 17 (JMP Statistical Discovery, Cary, United States) was used to perform One-way ANOVA tests.

Results and discussion
General sequencing metrics
Of the 49 sequenced samples, each was evaluated using the following four metrics: (1) total sequence reads generated per sample, (2) number of target reads from the source animal that furnished the parchment (i.e., the top non-human mitochondrial genome with the highest number of target reads), (3) percent of the ~16,000 base pair mitochondrial genome reference covered, and (4) depth of coverage (i.e., number of individual reads at a single position in the mitochondrial genome). When considering all four metrics regardless of sampling method, the Modern parchment was the best performer, followed by 15-311, 1785, and 1894 (Supplementary Tables 1–4). The Modern document, being a newer parchment and having endured less age-related damage, was expected to have higher amounts of DNA intact from the source species. The genetic material contained within a parchment document can be damaged by a variety of factors such as during the manufacturing process, designated use, environmental conditions, and storage[2,19,29,30], meaning age is not the only indicator of DNA quality. This

may explain why parchment 1894 (Supplementary Table 4) was a lower overall performer despite being the second youngest parchment. Consensus mitochondrial genome sequences of authenticated samples can be found at 10.6084/m9.figshare.29120510.

A comparison of each non-destructive sampling method was made by averaging the results for each the four metrics across all parchments (Table 2), given some variability within and between individual documents were observed (Supplementary Tables 1–4). The sampling method which resulted in the highest target reads was brushing with a 30 s eraser pre-cleaning. On average, brushing with an eraser pre-cleaning generated 15,184 target reads, 98% of the mitochondrial genome covered, and an average depth of 115X coverage. Across all three methods, samples subjected to a 30 s eraser pre-cleaning prior to collection resulted in higher target reads from the source animal and mitochondrial genome coverage compared to the same collection method without pre-cleaning, likely due to the removal of surface contaminants.

Across all parchment documents, no significant difference was observed in the depth of coverage across the mitochondrial genome when using two versus five lifts for either gecko tape (64X vs. 58X, respectively; $p = 0.7372$) or fiber lift tape (76X vs. 95X, respectively; $p = 0.6386$) (Table 2). Similarly, the average depth of coverage for fiber lift tape with five lifts only increased by 12X when treated with an eraser pre-cleaning (107X vs. 95X, respectively). Supplementary Tables 1–4 show the depth of coverage for each parchment document. While omission of the eraser pre-cleaning resulted in significantly more mapped human DNA than eraser pre-cleaned samples ($p = 0.0055$), there was no significant difference in the resulting depth of coverage due to pre-cleaning status ($p = 0.6046$). While the eraser pre-cleaning treatment successfully removed surface contaminants, such as human touch DNA that would otherwise take up space on a sequencing run, it may also be removing some of the "loose" cellular material of the authentic parchment. Further, while depth of coverage was significantly impacted by

Table 2 | Summary of processing metrics for new non-destructive methods

Non-Destructive Sampling Method	# Tape Lifts	Pre-Cleaning	Average # Reads	# Target Reads	% Mitochondrial Genome Covered	Average Depth of Coverage	% Human Contamination
Brushing	-	No	186,503	8800	99	59	32
	-	Yes	172,449	15,184	98	115	14
Gecko Tape	2	No	106,338	8567	87	64	13
	5	No	110,029	7772	92	58	31
	2	Yes	121,686	12,869	93	91	9
	5	Yes	85,880	8925	97	66	6
Fiber Lift Tape	2	No	111,297	9899	83	76	32
	5	No	136,332	12,074	78	95	24
	2	Yes	81,734	7770	79	64	13
	5	Yes	140,517	13,339	92	107	6

The average metrics across all four parchment documents are represented.

Table 3 | Top match species of sequence reads obtained from blanks

Blank type	Top match species	# reads
Brush only	Pig	14
Eraser crumbs only	Dog	194
Fiber lift tape only	Cattle	45
Gecko tape only	Cattle	285
DNA isolation	Cattle	*2

The top non-human match was selected to monitor contamination from sampling materials or wet-laboratory processing. Sampling blanks represent clean sampling material that did not come into contact with parchment (n, 1 each). The DNA isolation blanks (n, 4) were carried through the full DNA isolation process starting with an empty tube containing no sample or sampling materials. *denotes that two reads were mapped to the cattle reference for two different DNA isolation blanks.

the specific parchment document tested (Table 1) ($p < 0.0001$), it was not impacted by collection method ($p = 0.9065$) or number of tape lifts ($p = 0.9598$).

Human contaminant sequence recovery

The surface of parchment documents can contain human DNA from repeated touching with bare hands, which can be collected during non-destructive sampling methods. The percent of human contamination was calculated by comparing the number of human reads and the sum of all reads. Table 2 demonstrates the average percent of human contamination per sampling method. The sampling methods without an eraser pre-cleaning resulted in statistically higher percentages of human DNA contamination ($p = 0.0055$). The Modern parchment document had significantly lower percentages of human contamination than the other parchments ($p = 0.0043$). This is likely attributed to the Modern parchment being less exposed to human handling overall; this parchment was produced as a reference in 2010 and stored rolled when not in use. The 1894 parchment had the highest amount of human contamination and a distinguishable difference ($p = 0.0042$) between samples with and without an eraser pre-cleaning (Supplementary Table 4). All samples from this parchment that did not include an eraser pre-cleaning had higher percentages of human contamination compared to the samples treated to an eraser pre-cleaning which all fall below 30% of human contamination (Supplementary Table 4).

Non-human contamination assessment

Sampling blanks were sequenced to monitor possible contamination derived from sampling materials or the wet-laboratory processing (Table 3). While sampling blanks from previous studies had minimal reads as expected[3], the blank gecko tape sample (Table 3) had the highest number of

mitochondrial DNA sequence reads with 285 mapping to the cattle reference. These data suggest that animal products could be used in the manufacturing of gecko tape. Notably, we could not find details on the manufacturing process of gecko tape to confirm or refute this. Two samples from the 1894 parchment (gecko tape with two lifts only and fiber lift tape with two lifts only) also had cattle as their top non-human match, although the source species of this document was previously identified as sheep[3]. A single eraser crumb sampling blank had 194 reads that mapped to the dog mitochondrial genome. In previous studies from our group where eraser sampling of parchment was completed, no dog mitochondrial DNA sequences were generated in the sampling blanks[3]. While Table 3 indicates low level contamination across each of the sampling materials, the results from any given sampling blank do not meet the four authentication criteria required to report source species[3]. External contamination such as parchment-repairing glues and materials, commonly derived from animals[29], may be another explanation for generating reads not derived from the known parchment source species.

This study evaluated three non-destructive sampling methods for the collection of cellular material from the parchment source species, both with and without an eraser pre-cleaning. Although all sampling methods were able to generate full/near-full mitochondrial genomes from the known source species of the parchment above our required thresholds, brushing with a 30 s eraser pre-cleaning is the recommended method moving forward due to (a) the convenience of this method and (b) the increased recovery of source species cellular material while minimizing contaminants. While there was no physical damage or residue left on the parchment documents from either the gecko tape or fiber lift following sample collection (after inspection under a light microscope), we do not advocate using gecko tape or the forensic fiber lift tape as their sticky nature made them challenging to use. A limitation of this study was that only four parchment documents were available in our personal collections for sampling. While including additional more diverse parchment available in private and public collections would have provided data to more robustly evaluate the described methods, testing of cultural artifacts with new unproven methods would not have been acceptable to collection directors/curators. The use of brushing with an eraser pre-clean could be implemented in studies looking to maximize the recovery of source species cellular material prior to downstream DNA analysis. Researchers also looking to examine biological organisms associated with the parchment (e.g., human DNA from repeated touching, animal-based surface treatments, pathogens) could collect the crumbs from an eraser pre-cleaning step for downstream DNA analysis. Similarly, studies interested in holistically examining all cellular material associated from parchment (source species, bacteria, pathogens, humans, etc.) would likely want to avoid pre-cleaning.

Data availability

Data is provided within the manuscript or supplementary information files. Consensus mitochondrial genome sequences of authenticated samples can be found at 10.6084/m9.figshare.29120510.

Abbreviations

DNA   deoxyribonucleic acid

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Author contributions

L.D.D. performed all sample collections, was involved in all steps of sample processing and analysis, and drafted the manuscript. M.S. was involved in study conceptualization, sample processing and analysis, and edited the manuscript. K.A.M. was involved in study conceptualization, funding acquisition, supervision and editing of the manuscript. T.L., M.B., and I.L. were involved with funding acquisition, supervision and editing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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