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ArcHives—combined palynological, genomic and lipid analysis of medieval wax seals

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Abstract

Beeswax is a product of honeybees (*Apis mellifera*) and has been used extensively through time, especially as the primary component in medieval sealing wax for authenticating millions of documents. Today, these seals form large collections which, along with the historical information in the documents that the seals are attached to, could be a potential biomolecular archive for honeybees. Here, we investigate the possibility of obtaining biological information from medieval wax seals by performing a palynological and shotgun metagenomic analysis on eight medieval wax seal fragments. Our palynological results show that some pollen and fungal spores remain in the seals, albeit very little. Only one out of eight samples yielded enough DNA for sequencing. Moreover, only minor parts of the DNA reads could be taxonomically identified and were identified as plant and fungal DNA. These results demonstrate some potential for using wax seals as biological archives, but most importantly provides a framework for future studies, in addition to understanding further the degradation of seals as cultural heritage objects. We emphasize that future analyses should focus on other methodologies to retrieve data for historical context or alternatively improve molecular methods and screen sample collections broadly.

Keywords Sealing wax, Conservation, Heritage science, Beeswax, DNA, Palynology

Introduction

Beeswax seals were regularly used to authenticate written documents in the past and the high period of their use was during the Middle Ages in Western, Central and Northern Europe [1]. The seal, which is comparable to today's signature, was often the only proof of authenticity [2, 3]. The materiality, and materials of wax seals is an area of research with considerable potential. Possible avenues include seal composition and form, plus the humans who made and handled them. Research in this area has included fingerprints left on seals by people during the sealing wax manufacturing process, as well as during the actual act of sealing the documents themselves [3–5], and hairs left in the medium possibly belonging to the sovereign owner of the seals [6].

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Whereas parchment manuscripts have been deeply studied for the information they can provide on the Middle Ages, seals have often been neglected. Seals have not been widely incorporated into historical research, partly perhaps because they are not particularly well understood, with most extant seals remaining unrecorded [7]. Archival materials have been seen more as carriers of information, not whole objects, and seals are not just plain ephemera, but a handmade object with purpose [8]. Seals can be divided into two major categories: pendant seals (attached to documents with a e.g. strip of parchment or a silk string) and those that are directly impressed *en placard*, on a document [2], and are also known as applied seals [7]. Sigillography, the study of seals, became popular in the eighteenth century, and as a consequence, many private collections were gathered only then and later donated to libraries and museums [8].

Currently, large quantities of seals are stored in archives with only a presumed provenance or estimated date, or none at all, and the majority of them are unrecorded [7]. The biomolecular information of these seals can provide valuable knowledge about their origin, like their provenance based on the pollen originating from the beeswax [10, 11], or other potentially informative microfossils embedded in it. The main component of historical seals was beeswax, with natural resins to improve strength, plasticising materials (e.g. turpentine) and fillers (calcium sulphates, or chalk, calcium carbonates). Colouring was achieved with a variety of pigments, but the very earliest seals are uncoloured, so-called 'white seals' or *cera alba* due to their light colour, and composed predominantly of pure beeswax, with resin appearing as an additive from the late-twelfth century [7].

From an archival perspective, a specific and serious problem with white seals is their flaking and fragility which is a challenge for conservation, and research to establish the best methods for preserving these materials is needed [12]. Identifying the quantity and quality of different components in sealing wax is also vital for understanding the deterioration phenomena of beeswax in seals, as the additional materials (such as pigments) may affect the chemical composition of beeswax. By understanding the components, chemistry and physical structure of white seals, in tandem with how temperature changes, humidity and light affect their aging and degradation, conservation methods such as consolidation can be improved [1, 12, 13].

In this study, we experiment with medieval sealing wax, from the viewpoint that it could potentially serve as a dateable, localizable biomolecular archive for honeybees. Honeybees, *Apis mellifera*, produce wax in their wax glands to build their hives, the honeycombs, and honey and beeswax have been collected from both wild and domesticated bee colonies [14]. Beeswax is an extremely complex material and the complete composition of it is still not fully understood, but it is well reported in literature. The major components of beeswax are primarily (here expressed in weight) monoesters (35%), hydrocarbons (14%), diesters (14%), triesters (3%), hydroxymonoesters (4%), hydroxypolyesters (8%), free fatty acids (12%), acid esters (1%), acid polyesters (2%), free alcohol (1%) and unidentified material or impurities (6%) such as proteins, DNA and pollen [14–16]. Pollen can potentially be used as a geographical fingerprint for the wax, as honeybees forage within a certain radius from the hive [14]. Beeswax is considered chemically inert, and it is insoluble in water (hydrophobic), therefore the preservation of DNA, susceptible to hydrolysis [17], could potentially be excellent within wax and record information about past honeybees.

The current decline in honeybee populations worldwide has received attention in recent years [18, 19]. Therefore, the knowledge we could gain from the beeswax on past honeybees could potentially help to provide valuable information about the modern situation. Additionally, we looked for evidence that might be useful for considering the manufacture of the seals, the provenance of the materials and factors that might influence the degradation of seals in archives.

Materials and methods

Samples

In this study, we used a collection of historic sealing wax fragments to explore the potential of medieval seals as a biomolecular archive. We designed the analysis workflow from the perspective of using one sample to obtain maximum information about the seal. Our main methods were DNA extraction and pollen analysis on all of the samples to test our hypothesis and methods, in addition to exploring the lipid profile of the Herefordshire seal sample (DA177) due to its better known historical origin. A sample amount of 0.5 mg was taken from sample DA177 for the material characterisation to identify the building compounds of the seal based on the lipid profile: beeswax and possible other additives, such as resin, rosin or turpentine. For the DNA a sample amount of 50 mg was used (corresponding to the size of a rice grain), and the remaining pellet from the extraction was taken for palynological analysis.

All of the samples were from uncoloured, i.e. 'white seals' with primary components of beeswax and resin, and from pendant-type seals. Samples (7) DA115, DA117, DA118, DA119, DA120, DA121 and DA122 were incidental fragments kept from crumbling seals, but with no

Fig. 1. **a** The Herefordshire document and its seal, powder from which was analysed in this study (DA177). **b**. Close-up of the seal. (Photos by HARC, used with permission)

further context or provenance information. These samples were provided by The National Archives, UK, and although their provenance is unknown, their state of degradation and non-pigmented nature would suggest that they are medieval. Sample DA177 was collected crumbles directly associated with a seal attached to a deed (Fig. 1a, b) from Herefordshire Archive and Records Centre, with contextual information from the document locating it to Prior's Frome in the manor of Mordiford, Herefordshire, with a date of 1391 (HARC Sample ID: P82/9132.96).

Lipid extraction

After scratching the surface of the seal from DA177 using a solvent cleaned needle, a sub-sample (0.5 mg) was removed for analysis. The sample was spiked with 10 µg of C_{34} *n*-tetratriacontane and 10 µg of C_{25} *n*-pentacosanoic acid before extracting with DCM/MeOH (2:1, *v/v*, 3 × 3 mL) byultrasonication (10 min × 3). The supernatants were combined and solvent removed under a gentle nitrogen (N_2) stream at 40 °C. An aliquot (1/3) of the total lipid extract (TLE) was then derivatised for 1 h at 70 °C with 40 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Sigma Aldrich). After derivatisation, excess BSTFA was removed from the TLE aliquot using a gentle N_2 stream at 40 °C; the aliquot was then diluted in 50 µL *n*-hexane prior to analysis by HTGC-FID and HTGC/MS (see Supplementary Information: HTGC-FID & HTGC/MS).

DNA extraction, shotgun library preparation and sequencing

DNA was extracted using a novel extraction procedure developed to extract molecules and small particles embedded in wax. In addition, we also modified the binding buffer for silica based purification of the DNA to allow for a lower volume (details can seen in the supplementary material, Additional file 1: Table S1 and S2). Briefly, the procedure included dissolving the samples (ca. 50 mg) in xylene together with 40 µL of 90% glycerol and 10% EB buffer (10 mM Tris–HCl, pH 8.0) for one hour. Xylene was then removed and 200 µL of guanidinium hydrochloride (GuHCl) based extraction buffer was added together with 2 µL trypsin and incubated for 30 min at 37 °C. Samples were then flipped in an equal volume phenol:chloroform:isoamylalcohol (1:1:1), by rotation for 15 min at room temperature followed by centrifugation at 3000 g for 2 min. The aqueous supernatant was transferred to a new tube. Binding buffer was added and mixed and the DNA was purified using a Monarch silica spin column (NEB, US). DNA was eluted in 30 µL EBT buffer (10 mM Tris–HCl, 0.05% Tween20) and quantified on a Qubit 3.0 fluorometer (Invitrogen).

Subsequently, extracted DNA was built into Illumina shotgun libraries using established protocols for degraded DNA [20], with optimizations [21]. Libraries were screened for quantity by qPCR and amplified with Illumina dual index primers using AmpliTaq gold (Applied Biosystems, USA) according to Carøe et al. 2018 [20], but using 18 cycles for all samples in indexing. Amplified libraries were purified with SPRIbeads and analyzed on an Agilent 2100 Bioanalyzer. Only sample DA118 yielded a sequenceable library, i.e. with enough target library and negligible background noise. DA118 was subsequently sequenced on an Illumina Hiseq4000 in single read mode for 80 cycles, yielding 58,049,168 raw reads. Blanks were made for the extraction step, library step as well as PCR reactions. None of the blanks contained enough DNA to be sequenced. A detailed description of all procedures and buffer recipes can be found in the supplementary material.

DNA analysis

Bioinformatic data analysis was conducted on DA118 by employing broad screening methods. Firstly, the data were analysed using nf-core/eager v2.4.4 [22], to perform quality control of the input sequencing data, to analyse the content of human and honeybee DNA, and to obtain the taxonomic profile of the reads that did not map to the genomes of the aforementioned organisms. Quality

control processing of raw reads was conducted using the pipeline's default settings to remove sequencing adapters, trim low quality bases and filter out reads shorter than 30 bp. The resulting high quality reads were mapped to the human (*Homo sapiens* genome assembly GRCh38, RefSeq accession GCF_000001405.40) and honey bee (*Apis mellifera*, RefSeq accession GCF_003254395.2) genomes, using default settings but filtering out reads whose mapping quality was lower than 20. Analysis of ancient DNA-associated damage patterns and reference genome coverage statistics were performed using default settings.

Taxonomic profiling of unmapped reads with nf-core/eager was conducted using Kraken2 with default settings and activating the metagenomic complexity filter included as part of the pipeline [23]. The analysis was conducted using the kraken2_RefSeqV205_Complete_500GB database, which includes all eukaryotic, bacterial, archaeal and viral genomes available in release 205 of NCBI's RefSeq database, along with the non-redundant nucleotide sequences (nt), Plasmid and UniVec_Core sequences [24].

For the sake of comparison and gathering supporting evidence, we also used DIAMOND [25]. A DIAMOND database was built using all the entries available in the UniProtKB TrEMBL amino acid fasta file from release 2021_04 [26]. The accession to TaxID mapping information required for building the DIAMOND database was extracted from the fasta file headers, while the needed nodes.dmp and names.dmp files were downloaded from NCBI's taxdump archive from Nov 1st 2021. The database was built using DIAMOND's makedb command as described in the software's manual. Unmapped reads from sample DA118 were analysed using the built database and running DIAMOND on blastx mode.

Lastly, an additional taxonomic profile of the unmapped reads was obtained using Mgmapper [27]. The analysis was conducted using the script designed for single-end reads (MGmapper_SE.pl), disabling the QC processing steps as this had been previously done with nf-core/eager, eliminating PCR/optical duplicates and mapping the reads with best mode to the following databases in the order listed in the supplementary, Additional file 1: Table S6. The results obtained for the different databases were collapsed at the species level using the script MGmapper_classify.pl with default settings. Taxonomic profiling output data from all the aforementioned tools were processed and plotted using custom python scripts.

Pollen analysis

Seven pellets left from the DNA extraction were embedded in ethanol absolute (99.9%) prior transportation for palynological analysis. A few drops of silicone oil (AK 2000) were added, and the samples were placed in an oven at 60 °C for a few hours until the alcohol had completely evaporated. A drop of the sample and silicone oil was placed on a glass slide and studied under a transmitted light microscope at × 1000 magnification. This was done for all samples until there was only one drop of sample left in the container. All pollen types were photo-documented and types named (see SI Additional file 1: Table S8 for complete information).

Results

Lipids

Solvent extraction by ultrasonication yielded an 81% (0.41 mg) lipid recovery. HTGC/MS analysis of the total lipid extract (Fig. 2) demonstrates a lipid profile comprising a homologous series of odd-numbered saturated aliphatic *n*-alkanes (C_{25}–C_{31}) dominated by *n*-heptacosane ($C_{27:0}$, 2.3%, *w/w* of TLE). An even-numbered *n*-alkanoic acid series (C_{22}–C_{34}) was also identified and is largely dominated by *n*-tetracosanoic acid ($C_{24:0}$; 9.7%, *w/w*). The abundance and distribution of these compound classes, combined with the presence of fatty acyl monoesters (C_{40}–C_{48}) and hydroxyl fatty acid monoesters (C_{42}–C_{50}) with a characteristic profile, presents a distinct fingerprint that agrees with previous studies of *Apis mellifera* beeswax [16, 28, 29]. *n*-Alcohols are absent from the lipid profile but are commonly reported as minor components of beeswax [28]. Diesters were identified but not individually distinguished.

DNA

Initial quality controls of the extracts indicated poor preservation as no DNA was detectable using the Qubit fluorometer on any sample. Our qPCR screening of the libraries showed that out of the eight samples analyzed, only one sample (DA118) yielded enough library for sequencing (Fig. 3). This also showed that the library of sample DA121 had markedly higher Ct values than the baseline (negative controls), suggesting inhibition of the library preparation or PCR, possibly from compounds in the sample. Pre-sequencing analysis on the 2100 Bioanalyzer of the library of DA118 showed ultra short inserts consistent with poor DNA preservation (see Supplementary Information: extraction protocol). This was confirmed after sequencing by an average read length in trimmed data of 54 bp. The sequencing run for DA118 generated a total of 58,049,168 reads that were subsequently analysed with the nf-core/eager pipeline. The first step of the pipeline involved the quality control

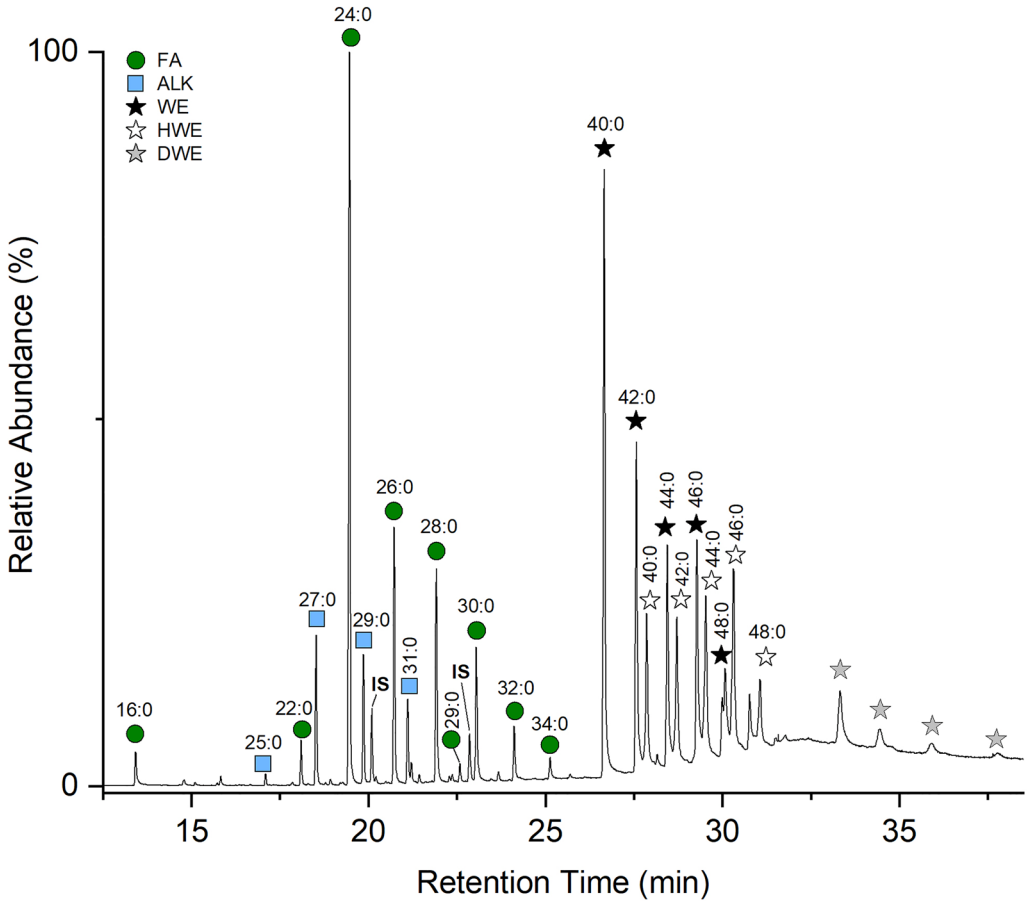

Fig. 2 Partial total ion chromatogram (TIC) of the TMS derivatised total lipid extract of sample DA177. Key: *n*-alkanoic acids (FA) are indicated by circles; *n*-alkanes (ALK), squares; fatty acyl monoesters (WE), black asterisks; hydroxyl fatty acyl monoesters (HWE), white asterisks and fatty acyl diesters (DWE), grey asterisks. The internal standards *n*-pentacosanoic acid ($C_{25:0}$) and *n*-tetratriacontane ($C_{34:0}$) are indicated by IS at 20.1 and 22.8 min, respectively. The acyl carbon number (*n*) and degrees of unsaturation (*i*) is indicated by *n:i*

processing of the input data that resulted in the elimination of 5,819,716 reads.

Mapping of high-quality reads to the human and honeybee genomes, two taxa that we expected would be dominant due to the nature of beeswax and the handling of the wax in processing, gave very few hits (13,448 for human and 5545 for honeybee, respectively). We therefore analyzed the remaining unmapped reads with three tools for broad taxa screening using DIAMOND, Kraken2 and MGmapper. The proportion of reads classified by each tool was 5.97%, 1.34%, and 12.1%, respectively. Although all tools reported that most of the classified reads were derived from members of domain Eukarya, there were very marked differences between the top 10 species reported by MGmapper and the other two selected tools. Kraken2 and DIAMOND were consistent in the identification of *Histoplasma capsulatum* and different species of the genus *Aspergillus* among the top 10 reported species, whereas the corresponding group reported by MGmapper was vastly comprised by butterflies from different families of the order *Lepidoptera* and the 10th spot was taken by one species of *Aspergillus* (Fig. 4). Thus, the only genus that all tools agree on is *Aspergillus*.

Binning the unmapped reads according to their length in groups of 5 bp intervals and screening the number of classified reads within each bin, revealed that for all tools the proportion of classified reads increased with the read length (Fig. 5). This clearly shows that longer reads, as expected, have a higher chance of being taxonomically identified. Moreover, we see that the peak at 80 bp, a result of inefficient adapterremoval, has a relatively higher number of hits. We hypothesise that this might be due to adapter contamination in the databases used, possibly explaining some of the unexpected taxa identifications such as butterflies.

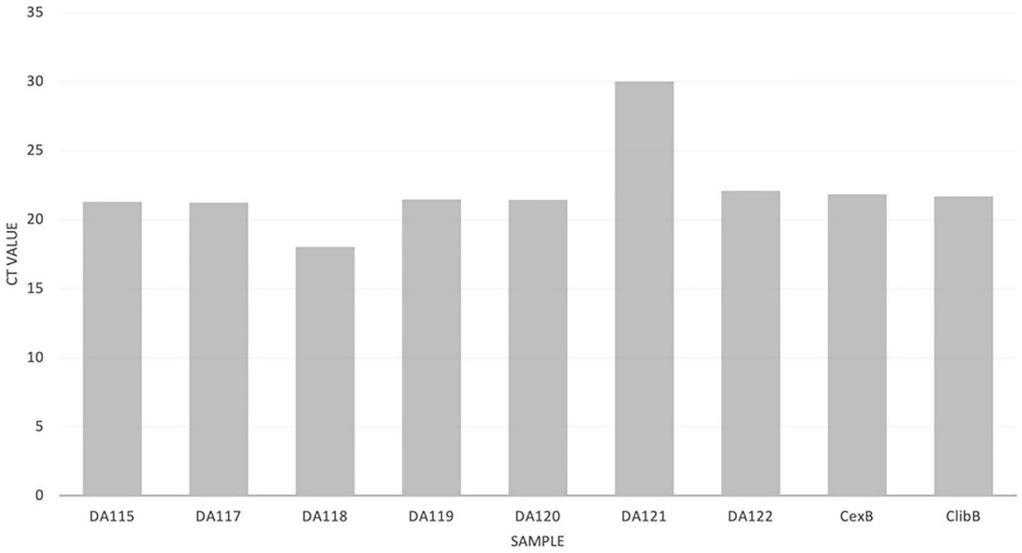

Fig. 3 Ct values for qPCR on non-indexed Illumina libraries. X-axis denotes sample names with CexB being extraction blank and ClibB being library blank

Pollen

Identifiable pollen was found in only two of the samples (DA122 and DA177), with the other samples being empty. The single pollen grain found in sample DA122 provided by The National Archives (TNA) was from the family Fabaceae cf. *Trifolium* (Fig. 6). The pollen (27 grains) found in sample DA177, the Herefordshire seal, was a combination of pollen types from trees, shrubs and herbs (Figs. 7, 8) that can be found on a dry meadow or as weeds on an agricultural field [30].

Another palynomorph was abundant throughout the rest of the samples: a small light brown spore (Fig. 7), the size of a spore from the fungus class of Hyphomycetes (including the genus *Aspergillus*). The spore did not have any characteristics that could identify it further. It was, nevertheless, numerously represented with between > 10 and > 1000 spores per slide, therefore a significant find in the samples. Full list of identified pollen can be found from the Additional file 1: Table S8.

Discussion

In our investigation for the potential for combined palynological and molecular analysis of medieval wax seals, we found that the results of the DNA and pollen analysis were limited, yet the lipid analysis could hold more potential for future work on a material such as sealing wax.

The lipid profile and abundance of compounds in DA177 agrees with previous research of *Apis mellifera* beeswax [16, 28, 29], thus providing unambiguous evidence for the use of beeswax in the seal. Although beeswax is typically resistant to large changes in chemical composition due to its hydrophobic nature, degradation may occur through exposure to UV, high temperatures or microbial attack [31]. As a result, modern and historic beeswax evidence different lipid profiles that may be characterised by the absence of *n*-alcohols (C_{24}–C_{38}) arising from the hydrolysis of wax monoesters; this is often accompanied by the loss of low weight *n*-alkanes [31, 32]. With this in mind, the absence of *n*-alcohols in DA177 may indeed suggest the sample is generally well-preserved although when compared with modern beeswax, the distribution of *n*-alkanes seems to favour higher-weight homologues as seen by the absence of *n*-tricosane (C_{23}) and the low abundance of *n*-pentacosane (C_{25}).

What is also interesting is the absence of terpenic acids in the sample, that presumably would be there due to the rosin traditionally added to the sealing wax mixture. However, this could indicate that the sample DA177 has either a very low, undetectable amount of rosin or none at all, where the latter fits in with previous knowledge on the composition of 'white seals' being mostly of beeswax [7, 15].

The use of organic and inorganic additives to modify the properties of beeswax is well-reported in wax seal recipes [33]. To harden and extend the working life of seals, pine resin (rosin) was historically added to beeswax and may be identified by pimarane and abietane acids [34]. Venice turpentine, an exudate of the European larch (*Larix decidua*) is also cited in wax seal recipes [35]. Like rosin, turpentine is typically dominated by abietane and pimarane acids but can be distinguished by the presence of larixol and larixyl acetate [36]. Shellac is another

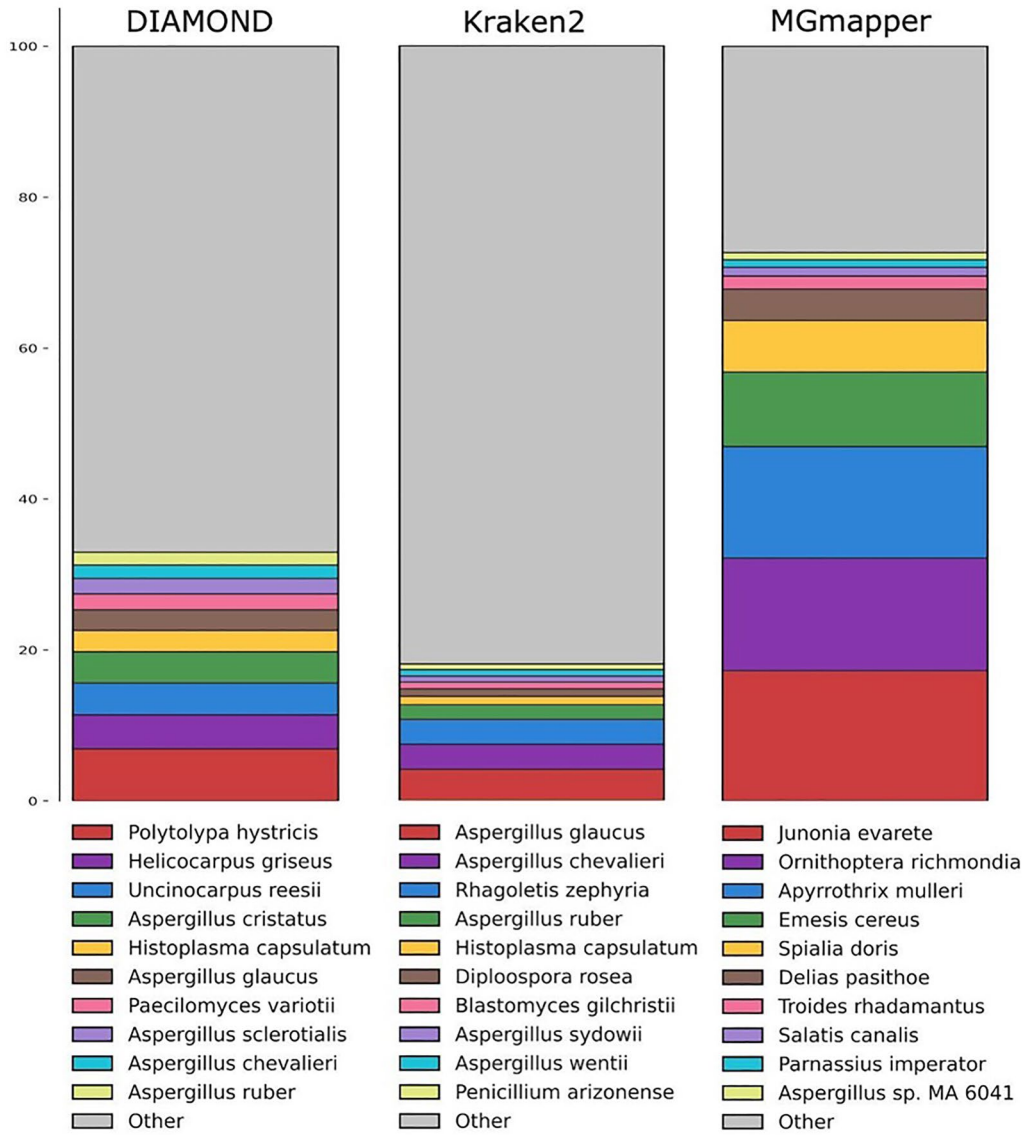

Fig. 4 Taxonomic profiling of reads using Kraken2, DIAMOND and MGmapper. Y-axis shows the percentage of reads assigned to each species. Unidentified reads are not included. The top 10 species with the highest amount of assigned reads by each tool are represented by an individual coloured bar, as indicated in the corresponding legends

common additive that originates from the *Kerria lacca* insect [37]. Its chemical composition differs from that of rosin and turpentine and is characterised by jalaric, laccijalaric, aleurtic and butolic acids. The absence of these biomarkers in DA177 suggests that these common admixtures were not employed during the production of the seal, instead, it is more likely the seal was manufactured from pure beeswax.

Overall, our analysis shows that the DNA content in the samples subjected to investigation was very low after extraction, and that only one sample (DA118) contained enough DNA to build a shotgun library. This could be a consequence of the efficient way that beeswax was refined (cleaned) by melting in hot water, allowing the water to cool and recovering purified wax floating on the surface. This method would be very efficient at stripping polar molecules (such as DNA and proteins) from the wax, because once the wax is melted, these molecules are free to dissolve in the water. Furthermore, at least one sample (DA121) very likely contained enzymatic inhibitors that affected the library preparation step, as evidenced by the obtained Ct value during the qPCR screening, which was higher than the corresponding value obtained for the negative

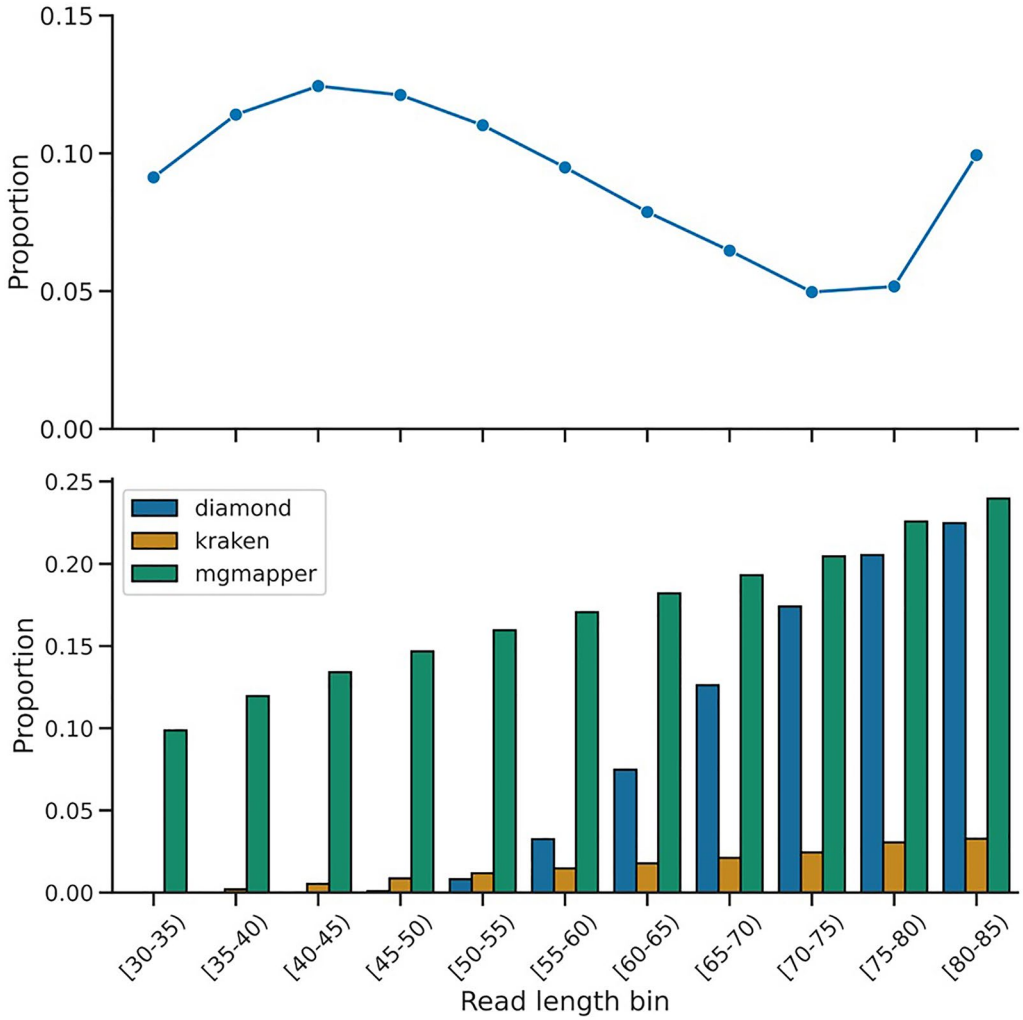

Fig. 5 Top shows proportion of reads with a given length after adapter removal. Note the increase at 80 bp, most likely caused by inefficient removal of adapter sequences (80 cycles single end Illumina chemistry was used for sequencing). Bottom shows the proportion of reads for read length intervals of 5 bases that could be classified using DIAMOND, KRAKEN2 and MGmapper

control (Fig. 3). This consequently restricted the sample from downstream amplification and sequencing.

The lack of enough DNA for sequencing in most of the analysed samples and the high proportion of ultra short sequencing reads in DA118 points to very poor conditions for DNA preservation in the wax seals, or to the overall low amount of bee-related DNA possibly present in beeswax to begin with. Consequently, this hampered the downstream taxonomic identification of sequencing reads. However, our analysis indicates that plant DNA can be sequenced from these samples, even if no pollen can be identified. It is striking that we find so little bee or human DNA, which would be expected from material that had been in contact with these taxa. As only refined wax is used for seals, this would explain how the wax is sterile from bee-related DNA. This ultimately questions whether our findings of plant and fungal DNA can even be traced to the original sample or is environmental contamination. If our findings of DA118 are representative for wax seal samples in general, it will prove difficult to obtain useful and reliable information through DNA analysis. Despite the high number of reads sequenced, the degraded state results in low success rate in taxonomic identification.

In addition, it should be noted that technical improvements such as extraction and library preparation could aid future studies on medieval wax samples. This could be especially relevant in terms of circumventing the effect of enzymatic inhibitors in the samples that could stem from wax additives such as plant resins as well as employing library methods with high efficiency and low background noise. Thus, while the

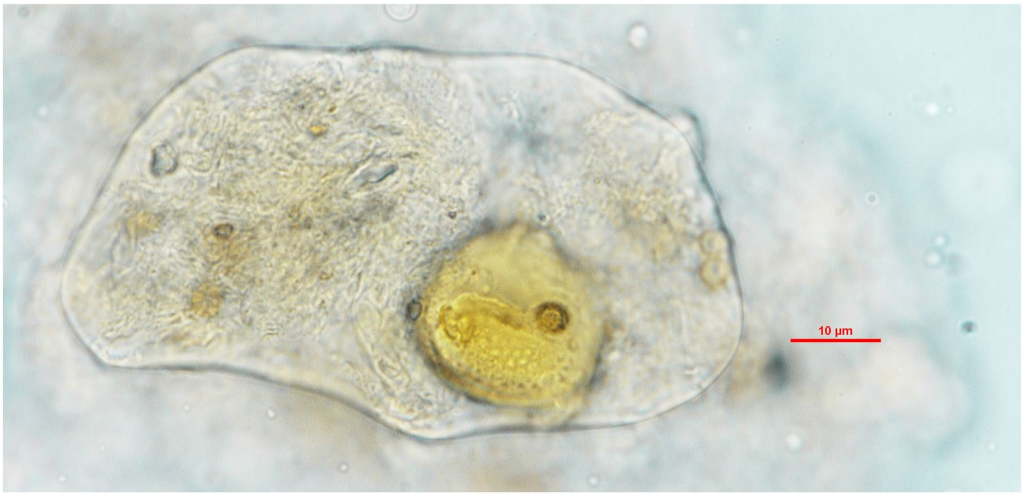

Fig. 6 Pollen from the family *Fabaceae cf. Trifolium* found in a wax-resin lump in sample DA122

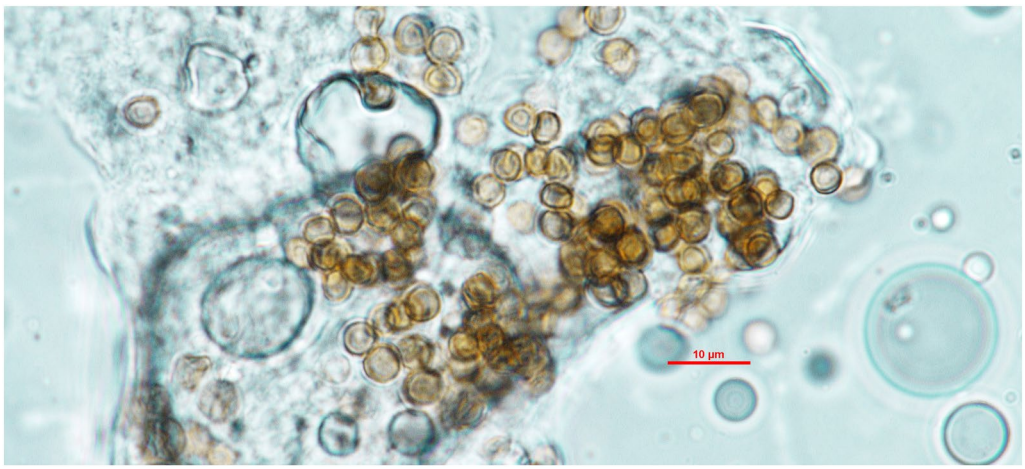

Fig. 7 Common small fungal spore from the sealing wax samples

prospects for future success of retrieving DNA from wax samples do not seem good, we emphasize that the present study relies on a small number of samples and that technological improvement could also help. When analysing ancient DNA from heritage objects such as medieval seals, it is imperative to extract and sequence relatively high amounts of DNA in order to capture the endogenous DNA that is present with much larger amounts of DNA from contaminants. Hence there is a need for efficient protocols for DNA extraction and library preparation.

Pellets after DNA extraction of the seals did not prove to be the best source of pollen for pollen analysis, yet the numerous pollen preserved in the Herefordshire sample, despite its small size, was an encouraging result. Additionally, the sample was not completely dissolved, and showed up as lumps in the sample slides, contributing to the challenges in identifying pollen from the samples. This residue could have been removed with a few steps following a pollen preparation protocol (acetolysis), that was not included in this study in order to prevent sample loss. Nevertheless, because of the lack of pollen preparation steps, the fungal spores were preserved in the pellets [38].

Overall, the samples yielded little pollen which could be due to several reasons. Firstly, the sealing wax material

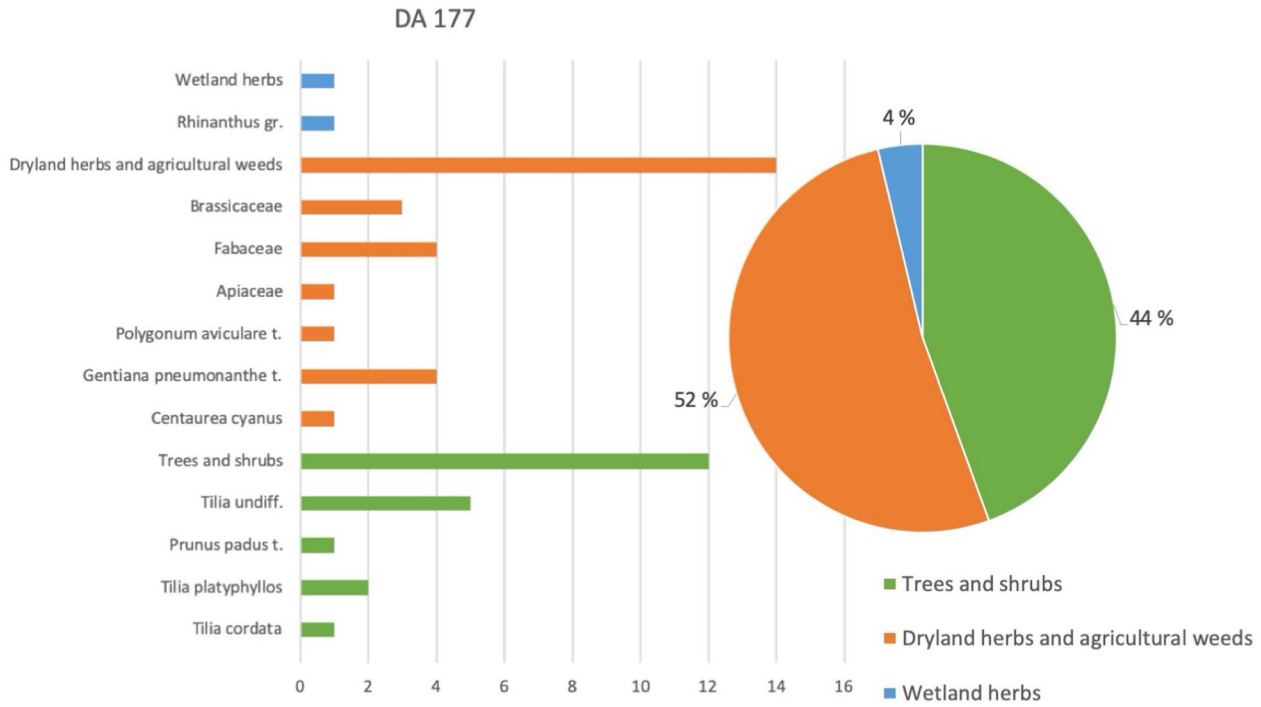

Fig. 8 Identified pollen taxa and the growth forms from sample DA177

could have been fairly sterile from pollen to begin with. Additionally, one or more steps in the procedure for DNA extraction could rid the sample of pollen, i.e. there may have been more pollen present in the sample originally but some of that may have been lost during the extraction process. There may have been a low concentration of pollen in the sealing wax, and the sample analysed was too small to detect it: during the setting of the warm wax, pollen may have been concentrated on certain areas [10], and this sample amount was too small to detect it. Despite these challenges, the information we found could prove to be useful and the potential of analysing a larger sample of the seals should be investigated in the future.

For example, the small fungal spore (group including *Aspergillus*) that was found in most of the seal samples could be an infection from storage in the archive. *Aspergillus* has been hypothesised as being one fungi responsible for the biodeterioration of sealing wax [1]. However, there is also a possibility that the fungal spores were accidentally or intentionally brought by the bees to the hive and embedded in the wax [39]. Are we analysing the micro-organism from the original source e.g. hive, the environment of the object or a part of the deterioration?

Additionally, investigating the high protein content of pollen [40] could bear an interesting approach to wax seals. However, as the proteins are inside the pollen, accessing the proteins would potentially require breaking the pollen grain with e.g. bead-beating, which would subsequently prevent the morphological identification of the pollen. Due to this and for the high accuracy of palynological identification, we prioritised the morphological identification of the pollen grains.

A material can go through either a chemical or a physical change during its ageing process. A chemical change can also act as a trigger for further mechanical damage, e.g. crystallisation breaking a structure [1]. The tendency of white seals to form a flaky, 'biscuit structure' is the main concern for conservators attempting to save the seals, as this makes the seals extremely prone to mechanical damage [41]. One of the causes of the deterioration of beeswax and the flaking or dryness phenomena is alterations in the crystallisation of the wax, leading to structural changes. As the polymorphic components change in the seal, this can lead to a more stratified structure and deterioration. Porosity can also be increased by the loss of hydrocarbons and volatiles during the ageing [8, 42]. This can also lead to the crystallisation of hydrocarbons to the surface of the seal, causing an effect known as "wax bloom" that may look like fungus [43, 44]. Analyses of the chemical composition of beeswax seals has revealed that these 'blooms' share nearly all their compounds with that of modern beeswax, interestingly however, when compared to historical wax seals the proportion of unsaturated hydrocarbons was found to be greater; this has

since led to the proposal that the migration of alkenes is indeed a causative mechanism for wax blooming [43].

In addition to this, previous suggestions for the lamellar structure of beeswax have been fungus-like bacteria, and the technique in which the seals were made: to incorporate all of the materials evenly to the mixture, it was kneaded by hand. When dealing with a natural product such as beeswax, it is always possible that even after thorough cleansing and filtering, some fine traces of pollen, bees and honey are left in the wax. Microorganisms, such as the wax-decaying bacteria, can feed on these in addition to the hydrocarbons of the wax, causing microbiological degradation. Some notes on the appearance of micro-organism, especially on the deteriorated surface, have been made and suggested as the clear cause of the deterioration [13, 15]. Our findings of *Aspergillus* sp. in both spores and as identified DNA, may support the hypothesis of fungi being a critical part of the biodeterioration mechanism of seals, yet to confirm this a wider set of samples should be analysed in tandem with the lipidic composition.

Conclusion

The samples used in this study consisted mostly of residual fragments collected during the conservation of seals. As a methodological development study, these fragments may not have been sufficient to provide a broad sample-set, and it should be noted that the DNA content could be vastly different as a result of wax production procedures, wax composition and storage conditions. Despite the low molecular success rate and the small sample number, we note that a similar analysis on other samples might prove successful. In the light of our results, our hypothesis of using sealing wax as a dateable, localizable biomolecular archive for honeybees, unfortunately does not stand. Yet, even crumbles of medieval seals can produce pollen results, and the lipid analysis can verify the material components of the seal. These results indicate that a systematic sampling and analysis of the lipids and pollen in sealing wax samples could provide more interesting data to study e.g. historical or biological questions. Although the pollen quantity is low, some information might be gained about honeybee foraging as a provenancing fingerprint and/or about the storage conditions of the seal. Furthermore, DNA analysis on medieval wax specimens seems to be very limited, not only due to the poor preservation of DNA, but also the composition of taxa and limited usefulness of this. We therefore suggest further work with this type of material, as there is still more potential for unlocking the unseen archives of medieval wax seals.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40494-022-00848-6.

Additional file 1: **Table S1**. Extraction buffer. **Table S2**. Binding buffer. **Table S3**. Glycerol buffer. **Table S4**. Washing buffer for silica purification. **Table S5**. EBT buffer 50 mL (for elution, dilution etc). **Table S6**. List of databases for MGmapper analysis. **Table S7**: Compounds identified in DA177 following analysis by HTGC/MS (Figure 2). Key: FA: n-alkanoic acids; ALK: alkanes; fatty acyl monoesters (WE); hydroxyl fatty acyl monoesters (HWE) and fatty acyl diesters (DWE). The internal standards: n-pentacosanoic acid (C25:0 FA) and n-tetratriacontane (C34:0 ALK) are denoted as IS. The acyl carbon number (n) and degrees of unsaturation (i) is indicated by n:i.

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Author contributions

The author's contributions were as follows: MJC planned the study. TMK led the overall paper and contributed to sample preparation for analyses. RE performed the palynological analysis. SJ performed the GC–MS analysis, interpretation and reporting. MR and CC conducted the laboratory work for the DNA analysis. GR-P and CC generated and analysed the DNA data. AS helped to acquire samples from HARC and helped to write the paper. LVA provided the TNA samples and helped to write the paper. MR-S and MJC helped to write the paper and contributed to the design of the study. AJT and CC designed the methodological part of the study. CC led the scientific concept of the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Data access will be added upon the acceptance of the manuscript. For the purpose of open access, MJC has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

Declarations

Competing interests

All authors declare no competing interests.

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