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IDENTIFICATION OF ANIMAL ADHESIVES USING DNA AMPLIFICATION

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Abstract

The aim of this study was to examine whether DNA was degraded in the manufacturing of animal glue. To test this, two different types of sturgeon glue (Acipenser sp.) were manufactured using historic recipes. One glue was boiled for a substantial amount of time and the other was kept under 75℃. DNA samples were collected from both glues in order to test whether the DNA was degraded in the heating process of making the glue. It was also tested whether two different kinds of flex canvas (for paintings), one coarse and one fine weaved would inhibit the PCR reaction. To do this the glue were applied onto the canvas and samples were collected. To examine the sample size needed to get an amplifiable DNA sample, different sizes were collected of the canvas, 1.0cm^2; 0,5cm^2 and 0,5cm of a single thread. It was possible to get amplifiable DNA in 11 out of 12 samples collected after the manufacturing of the glue and in 18 out of 24 samples collected of the canvas. In four out of the five cases where it was not possible amplify DNA, the sample belongs to the smallest size of the canvas investigated. As shown in this study it is possible to get DNA out of boiled animal glue and glue applied onto canvas. The application of a DNA techniques provides several new possibilities for further material analysis of (pre)historic artefacts.

Keywords: *Animal adhesive; glue; Sturgeon glue; Organic binding media; Isinglass; Acipenser sp.; Huso sp.; Cytochrome b; mtDNA*

Introduction

Correct identification of materials used in the manufacture and later restoration of historic objects and artwork is important for curators, conservators and art historians. Several materials used in conservation are incompatible and will in time, destroy, rather than preserve the object [1]. Identification of the original materials will provide information necessary for a precise reconstruction of historic manufacturing techniques and for a well-judged future preservation of the object [2].

Animal glue, consisting of the structural protein collagen derived from animal bones or hides have been used for thousands of years in the manufacturing of objects and artwork. Before the advent of synthetic polymers, it was one of the strongest adhesives known. In

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conservation and restoration of cultural objects, animal derived binders have been used as an adhesive (glue), for consolidation, filler for pigments and as varnish [1]. In this study, we wanted to investigate the degradation of DNA during the manufacturing process of sturgeon glue (Isinglass). Sturgeon glue was chosen because it is the purest form of animal adhesive (Containing very little extraneous material [3]) and has been widely used by artists from the time of ancient Egypt (1500 BC) to twentieth century France [4]. In conservation, sturgeon glue has been used since the seventeenth century [3].

To identify organic compounds in historic objects, earlier studies have primarily been based on gas chromatography-mass spectrometry (GC-MS), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry or Fourier Transform Infrared Spectroscopy (FTIR) performed on microscopic samples of e.g. layers of paint [5-7]. While these methods are good for identifying organic compounds such as proteins, drying oils, wax, plant resins and polysaccharide gums they cannot provide detailed information on the origin (species and haplotypes) of the products (Although see [8] who claim to be able to identify species-specific peptides in animal adhesives). To identify this, one needs to study the genetic code, DNA.

The first reports of a DNA being extracted relied on bacterial cloning to amplify small sequences of mitochondrial DNA [9, 10]. With the invention of the polymerase chain reaction (PCR) and second generation sequencing [11], ancient DNA research has progressed from the retrieval of small fragments of mitochondrial DNA to large scale studies of ancient populations and even complete mitochondrial as well as nuclear genome sequences [12-14]. The PCR technique is an extremely sensitive method that can detect minute amounts of specific DNA molecules and amplify these molecules billions of times in a few hours.

The first report of successful DNA identification of the species of fish from which sturgeon glue (isinglass) was manufactured date to 1995 [15]. DNA in animal glue used as a consolidant of archaeological bones has been described as a source of contamination [16]. Unfortunately, the perspectives for historic and conservation research in this discovery were largely ignored. A recent paper has reported the first identification to species level of animal glue used as a binding layer for pigments on a ~1415 A.D. polychrome terracotta figure by Donatello [17]. Unfortunately, the samples were taken from detaching flakes, which makes the results somewhat unreliable.

In this study, we wanted to investigate if the manufacturing process of animal (sturgeon) glue did affect the survivability of the DNA. Several factors are know to decay the DNA molecule; salt content, radiation, pH, oxygen and free water. But it is temperature which is believed to be the most degrading factor [18, 19]. Therefore manufacturing procedures were chosen that represent various extremes expected to degrade the DNA (temperature, boiling time, inhibitors of the PCR reaction) and old original recipes dating back to the middle of the 1900 century were followed. This project will describe the amount and quality (measured in base pairs, bp) of PCR amplifiable mitochondrial DNA (mtDNA) only.

Materials and methods

Production of fish glue

In this study we used dried swim bladders from sturgeon (*Acipenser* sp. and *Huso* sp.) in the production of two different fish glues. The swim bladders had been stored at the School of Conservation in dry condition since it was purchased from a Russian trader in 1992. The first glue (Glue1) was produced using the "Isinglass glue" recipe by Chemist [20]. Two third of one bladder was cut off and dissolved in 50mL of water while slowly heated. After one hour of boiling an additional 25mL of water was added. After one and a half hour of boiling all larger undissolved pieces of bladder were removed with a spatula and a small amount of the liquid glue was poured onto an aluminium foil and dried in an oven at 30°C over night. The rest of the glue was placed in a sterile container and stored in a refrigerator at five degrees centigrade. The second glue (Glue2) was produced using the "Tierissche Leime" recipe from Doerner [21]. Again two third of another bladder was cut off and softened in 50mL of water at ambient temperature throughout the night. The softened bladder was then heated using a water bath with the temperature below 75°C. After two hours an additional 35mL of water was added. After four hours all undissolved pieces of bladder were removed with a spatula and the same drying procedure as for the first glue was followed.

Glue on canvas

After seven months storage at five degrees centigrade the two glues were reheated. The first one (Glue1) was heated to 100°C and the second glue (Glue 2) was placed in a water bath and carefully monitored, so it did not boil. Two different kinds of canvas known to have been used by painters in the 1800s were chosen (pers. com. Mikkel Scharff). Both canvases were made of flex, but weaved with different size of thread, one fine (0.05mm) and one coarse (0.9mm). This was done to test whether there was a difference in the DNA yields when using different fabrics for painting and to see whether the flex canvas would inhibit the PCR reaction. To test whether the application of the glue made a difference, a thin layer was applied with a marten hair paintbrush and a thicker layer was applied with a sterile knife onto each of the two the canvases.

DNA samples

All DNA work was conducted at the School of Conservation and at the Natural History Museum in Copenhagen, Denmark. Standard precautions were taken to minimize the risk of contamination [12]. DNA samples were collected from the two differently produced glues while they where still liquid as well as a sample was collected after the glue had been dried. To collate the results a standard sample were also collected of the two dried raw sturgeon bladders. After the glue was applied to the canvas, it was left to dry for 4 days (96 hours) at ambient temperature before the DNA samples were selected. The samples were collected as a 1.0cm^2, 0.5cm^2 of canvas and 0.5cm single thread. In total 24 samples were collected of the two canvases. DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN) following the supplier's manual. Two sets of blank control samples were made; one purification blank and one PCR blank. Different laboratories for making the glue, extracting the DNA and running the

PCR were used. Two different sets of *Acipenser* sp. primers targeting cytochrome *b* were used: One designed to amplify a relatively long fragment length (app. 212 bp) and another amplifying a shorter fragment length (app. 86 bp). The first DNA extraction was done with the short primer in order to determine whether any DNA was preserved in the glue. The longer primer was used to ascertain if longer fragments of DNA were also preserved.

DNA Amplification

A standard double-stranded 50µl PCR was carried out using one µl of the extracted DNA as template. The first selected primer was designed to amplify a fragment of app. 86 bp on the cytochrome *b* gene. The forward primer H15392-Acip-for: (5'-GAY AAR GTA ACA TTC CAC CC-3') and the reverse primer H15497-Acip-rev: (5'-TRA AGT TGT CTA AAT CGC -3') [22]. The DNA samples collected of the glue and the raw bladder were set up for thermal cycling on an ESCO swift MinePro, Buck & Holm. The PCR-conditions were: One initial cycle of denaturation (95°C for 15 minutes), followed by 35 cycles (95°C for 30 seconds, 43°C for 40 seconds and 72°C for 30 seconds) and finally 5 minutes extension step at 72°C. As a TEMPase HotStart Maser Mix (VWR) was used for the PCR setup, the 15 minutes at 95°C was needed to activate the enzyme in the mix. The second set of primers targeting 212 bp on cytochrome *b* were Ancipen1-for: (5'- CAG GTT TCT TTT TGG AGG T-3') and the reverse primer Ancipen2-rev: (5'-ACA CAA ATC YTA ACA GGA CT -3') [23]. The PCR-conditions were the same as for the first primer set, except the annealing temperature was decreased to 42°C.

The DNA samples collected from the canvas were also set up on the same PCR machine as previously used and a TAQ DNA polymerase (WVR) and SET DNTP 100MM DA DC DG DT (WVR) was mixed, the PCR condition for the primers H15392-Acip-for and H15497-Acip-rev were: one initial cycle of denaturation (94°C for five minutes), followed by 55 cycles (94°C for 45 seconds, 56°C for 30 seconds and 72°C for 45 seconds) and finally 4 minutes extension step at 72°C. The thermal cycling with the primers Ancipen1-for and Ancipen2-rev were: one initial cycle of denaturation (94°C for seven minutes), followed by 35 cycles (94°C for 40 seconds, 55°C for 30 seconds and 72°C for one minute) and finally seven minutes extension step at 72°C.

DNA sequencing

The PCR products were visualised on a two percent agarose gel containing GelRed Nucleic Acid Stain (BIOTIUM). The PCR products were purified using QIAquick® purification kit (QIAGEN). The DNA concentrations were measured using an Eppendorf Biophotometer, where five µl of the template were mixed with 45µL sterile H_2O. The purified PCR products were then used as templates for a 12µL cyclic sequencing reaction using ABI prism® BigDye® Terminator v1.1 Cycle Sequencing kit. For the cyclic sequencing reaction the same primers were used as for the two initial PCR reactions and the PCR conditions were as follows, one initial cycle of denaturation (96°C for three minutes), followed by 30 cycles (96°C for ten seconds, 50°C for 15 seconds and 60°C for four minutes). The cyclic sequencing PCR was carried out on a gradient Cycler, BIO-RAD, DNAEngive, Peltier Thermal Cycler. The purified sequencing products were run on a HITACHI 3130xl Genetic Analyser Applied Biosystems with an attached computer with Genetic Analysis Program. The sequences were

assembled using the computer program CLC Main Workbench 6 and submitted to NCBI nucleotide collection for comparison of the sequences with those at NCBI.

Results and discussion

Manufacturing of the glue

In table one the sequencing results from the samples collected right after the manufacturing of the glue and of the raw swim bladders are shown. It was possible to species identify 11 out of 12 samples however the one sample we could not identify was from the glue which had been boiled and using the primer amplifying the longer fragment. The same glue was later reheated and applied on canvas before samples were collected and sequenced with positive species identification as a result.

The DNA concentrations were measured after the PCR purification. Looking at the concentration measurements of the boiled glue, the concentrations are higher when using the primer amplifying a shorter fragment than when using a primer amplifying a longer fragment indicating that the DNA molecules might have undergone degradation in the heating process of the manufacturing of the glue.

After sequencing the samples it was clear, that the two different swim bladders used in the experiment were from two different species of sturgeon. One from the European sturgeon, *Huso huso* L. and the other were either from a Siberian sturgeon *Acipenser baerii* Brandt or a Russian sturgeon *Acipenser gueldenstaedtii* Brandt & Ratzenburg, however the sequenced part of the mitochondrial DNA could not be used to separate the two species (see later).

A control sample containing all the PCR components and a blind sample containing the purification and PCR components both showed no sign of DNA ensuring that the samples had not been contaminated.

Table 1. The sequencing results of the 12 samples collected after the manufacturing of the glue and of the raw sturgeon swim bladders.

Sample no.	Type of glue	Sample description	DNA concentration (μg/μL dsDNA)	Species identified	Bases identities	Gaps
Primer: Ancipen1-for and Ancipen2-rev, 212 basepair						
LIM2-10-1-2	Glue 2	Liquid glue	43	*H. huso*	208/209	0/209
LIM2-10-2-2	Max temp. 75°C	Dried glue	100	*H. huso*	211/212	0/212
LIM2-10-3-2	-	Raw bladder	74	*H. huso*	209/210	0/210
LIM2-11-4-2	Glue 1	Liquid glue	37	-	-	-
LIM2-11-5-2	100°C	Dried glue	39	*A. baerii/ gueldenstaedtil*	211/212	0/212
LIM2-11-6-2	-	Raw bladder	46	*A. baerii/ gueldenstaedtil*	211/212	0/212
Primer: H15392-Acip-for and H15497-Ancip-rev, 86 basepair						
LIM2-10-8-2	Glue 2	Liquid glue	32	*H. huso*	62/62	0/62
LIM2-10-9-2	Max temp. 75°C	Dried glue	40	*H. huso*	62/62	0/62
LIM2-10-10-2	-	Raw bladder	52	*H. huso*	82/83	1/83
LIM2-11-11-2	Glue 1 100°C	Liquid glue	52	*A. baerii/ gueldenstaedtil*	82/83	1/83
LIM2-11-12-2		Dried glue	77	*A. baerii/ gueldenstaedtil*	71/71	0/71
LIM2-12-13-2	-	Raw bladder	64	*A. baerii/ gueldenstaedtil*	83/83	0/83

Glue on canvas

Out of the 23 samples in total, 18 samples were species identified. Out of the 5 samples, which were not identified, 4 of them were collected as a 0,5cm of thread and the last one as a 0,5cm^2.

There were no indications of differences in the DNA outcome whether the primer targeting a small fragment or a longer fragment was used.

The results indicated that there were no PCR inhibitors in either the manufacturing or in the canvas itself. Not surprisingly the smaller sample size, the less likelihood of an amplifiable DNA sample.

Table 2. Sequencing results of the glue on canvas

Sample no.	Type of glue and sample description	Sample size	DNA concentration (µg/µl dsDNA)	Species identified	Bases identities	Gaps
Primer: H15392-Acip-for and H15497-Ancip-rev 86 basepair						
AME12-1-2	Fine weaved, Glue1 (boiled), brush	1 cm^2	60	*A. baerii/ gueldenstaedtil*	23/23	0/23
AME12-2-2		0,5 cm^2	61	*A. baerii/ gueldenstaedtil*	66/67	0/67
AME12-3-2		0,5 cm thread	57	-	-	-
AME12-4-2	Fine weaved, Glue2 (water bath), brush	1 cm^2	61	*H. huso*	71/71	0/71
AME12-5-2		0,5 cm^2	67	-	-	-
AME12-6-2		0,5 cm thread	65	*H. huso*	73/76	1/76
AME12-7-2	Coarse weaved, Glue1 (boiled), brush	1 cm^2	74	*A. baerii/ gueldenstaedtil*	84/84	0/84
AME12-8-2		0,5 cm^2	69	*A. baerii/ gueldenstaedtil*	81/82	1/82
AME12-9-2		0,5 cm thread	71	*A. baerii/ gueldenstaedtil*	79/79	0/79
AME12-10-2	Coarse weaved, Glue2 (water bath), brush	1 cm^2	71	*H. huso*	83/85	1/85
AME12-11-2		0,5 cm^2	69	*H. huso*	83/84	1/84
AME12-12-2		0,5 cm thread	71	*H. huso*	77/79	2/79
AME12-13-2	Fine weaved, Glue2 (water bath), knife	1 cm^2	76	*A. baerii/ gueldenstaedtil*	21/21	0/21
AME12-14-2		0,5 cm^2	77	*A. baerii/ gueldenstaedtil*	72/73	1/73
AME12-16-2	Fine weaved, Glue2 (water bath), Knife	1 cm^2	83	*H. huso*	77/78	1/78
AME12-17-2		0,5 cm^2	81	*H. huso*	22/22	0/22
AME12-18-2		0,5 cm thread	75	-	-	-
AME12-19-2	Coarse weaved, Glue2 (water bath), Knife	1 cm^2	81	*A. baerii/ gueldenstaedtil*	74/74	0/74
AME12-20-2		0,5 cm^2	85	*A. baerii/ gueldenstaedtil*	37/37	0/37
AME12-21-2		0,5 cm thread	88	*A. baerii/ gueldenstaedtil*	32/32	0/32
AME12-22-2	Coarse weaved, Glue2 (water bath), Knife	1 cm^2	87	*H. huso*	29/29	0/29
AME12-23-2		0,5 cm^2	85	*H. huso*	73/74	0/74
AME12-24-2		0,5 cm thread	88	*H. huso*	82/85	1/85
Primer: Ancipen1-for and Ancipen2-rev 212 basepair						
AME12-1-3	Fine weaved, Glue1 (boiled), brush	1 cm^2	80	*A. baerii/ gueldenstaedtil*	212/214	1/214
AME12-2-3		0,5 cm^2	78	*A. baerii/ gueldenstaedtil*	212/213	0/213
AME12-3-3		0,5 cm thread	79	-	-	-
AME12-4-3	Fine weaved, Glue2 (water bath), brush	1 cm^2	85	*H. huso*	213/214	0/214
AME12-5-3		0,5 cm^2	84	*H. huso*	24/24	0/24
AME12-6-3		0,5 cm thread	84	-	-	-
AME12-7-3	Coarse weaved, Glue1 (boiled), brush	1 cm^2	84	*A. baerii/ gueldenstaedtil*	212/214	1/214
AME12-8-3		0,5 cm^2	84	*A. baerii/ gueldenstaedtil*	212/213	0/213
AME12-9-3		0,5 cm thread	84	*A. baerii/*	212/214	1/214

				gueldenstaedtil		
AME12-10-3	Coarse weaved, Glue2 (water bath), brush	1 cm^2	84	*H. huso*	213/214	0/214
AME12-11-3		0,5 cm^2	84	*H. huso*	212/213	0/213
AME12-12-3		0,5 cm thread	86	*H. huso*	124/134	1/134
AME12-13-3	Fine weaved, Glue1 (boiled), knife	1 cm^2	84	*A. baerii/ gueldenstaedtil*	212/213	0/213
AME12-14-3		0,5 cm^2	103	*A. baerii/ gueldenstaedtil*	175/180	2/180
AME12-16-3	Fine weaved, Glue2 (water bath), Knife	1 cm^2	90	*H. huso*	213/214	0/214
AME12-17-3		0,5 cm^2	87	*H. huso*	212/215	1/215
AME12-18-3		0,5 cm thread	89	-	-	-
AME12-19-3	Coarse weaved, Glue1 (boiled), Knife	1 cm^2	89	*A. baerii/ gueldenstaedtil*	212/217	4/217
AME12-20-3		0,5 cm^2	90	*A. baerii/ gueldenstaedtil*	48/48	0/48
AME12-21-3		0,5 cm thread	92	*A. baerii/ gueldenstaedtil*	138/141	1/141
AME12-22-3	Coarse weaved, Glue2 (water bath), Knife	1 cm^2	95	*H. huso*	214/216	1/216
AME12-23-3		0,5 cm^2	105	*H. huso*	213/215	1/215
AME12-24-3		0,5 cm thread	115	*H. huso*	213/214	0/214

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Conclusion

As it was possible to species identify all but one sample right after the manufacturing of the indicates that the boiling process do not degrade raw organic material in a matter which makes it impossible to amplify a DNA sample. However the concentration measurements indicated that there were a higher amount of shorter fragments after the glue was boiled.

We were only able to species identify one of the swim bladders when using the primers we did. As genetic contamination of *A. gueldenstaedtii* with *A. baerii* or occurrence of hybrids of the two species has made the identification between these two species difficult [24].

The samples collected of the canvas indicated that there were no PCR inhibitors in the canvas and that the method in which the glue was applied made no difference in the DNA outcome. The smaller sample there was collected, the less likely it was to get an amplifiable DNA sample. Even with a relatively long storage time (seven months) from the glue was made until it was applied onto the canvas did not affect the possibility of amplifiable DNA samples.

Perspective

As shown in this study it is possible to get DNA out of boiled animal glue and glue applied onto canvas. The application of a DNA techniques provides several new possibilities for further material analysis of (pre)historic artefacts: The main biological question, what animal/plant an object derives from can be answered on a taxonomic level. Even the individual animal can be identified. DNA-profiling shows whether two finds belong together (i.e. were made from the same individual animal). At the same time, it can tell us where and when this original animal came from. This could also be used to expose forgeries. The comparison of the genetic status of a historical animal or plant with other historical or recent data will increase our knowledge not only about the material itself but also about domestication, cultivation, planting and herding practices. This information is unattainable using traditional chemical methods.

Personal communication

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