Journal of Archaeological Science 181 (2025) 106305

Contents lists available at ScienceDirect

Journal of Archaeological Science

journal homepage: www.elsevier.com/locate/jas

Identify the genus origin of animal glue used in Chinese historical mortars using a new DNA mini-barcoding method

Qian Wu [a], Bingjian Zhang [a,b], Qiong Zhang [c], Mingzhi Ma [d], Yulan Hu [a,*]

[a] Department of Cultural Heritage and Museology, Zhejiang University, Hangzhou, 310028, China
[b] Department of Chemistry, Zhejiang University, Hangzhou, 310027, China
[c] The Palace Museum, Beijing, 100000, China
[d] Shaanxi Academy of Archaeology, Xi'an, 710054, China

ARTICLE INFO

Keywords:
Ancient DNA
Animal glue
Historical mortars
Species identification
DNA micro-barcoding

ABSTRACT

In historic buildings and paintings, animal glue was one of the first and most used organic binders. The identification of these binders would provide a clue for the life of the ancient human beings. However, the precise detection of the clue is challenging as the amount of the glue is very low and always mixed with other impurities. Previously, the ELISA method was successfully used in the identification of the glue in family level. However, the method can not tell the species of the glue. For example, it can not known whether the glue was from cattle or sheep using ELISA methods. This study provides a novel biological technique to identify the species of animal glue used as ancient binders. Hence, the accurate identification results at the species level can be obtained by DNA barcoding method with low detection limits. Moreover, ten animal glues belong to different species can be identified using a pair of universal primer at one time. These species include pigs, cattle, buffalo, goats, sheep, camels, deer, dogs, horses, and rabbits. Actually, the species of animal glue used in mortars obtained from three cultural heritages, the Neolithic Yulin City in Shaanxi Province, the Forbidden City in Beijing, and the Confucian Temple in Qufu, Shandong Province, China, were successfully identified. It was demonstrated that certain animals were not only consumed as food resource at that time, but their leftover bones, skins, and other parts were also utilized to produce animal glue. Additionally, our findings is consistent with the records in ancient books, which documented the species of animals frequently used in the manufacturing of animal glue. This is the first report using DNA barcoding method for the identification of animal glue in historic mortar. The identification of animal glue's species can not only help archaeologists understanding the agriculture and animal husbandry in certain area, but also can help people find the suitable materials for restoration and conservation of cultural heritage.

1. Introduction

Animal glue is a brown (or pale yellow) amorphous protein solid derived from animal fur, bones, tendons, and other tissues.As one of the oldest and most widely used organic binders, it has played a crucial role in architectural plasters, mural paintings, and ancient pottery decoration (Fang, 2014; Zadrożna et al., 2003). Archaeological evidence demonstrates its global use across various civilizations.Archaeological evidence demonstrates its global use across various civilizations.For instance, murals depicting the production and application of animal glue have been discovered in Egyptian pyramids dating back to 4000 BCE (Sweatt, 1946).Similarly, in Mongolia's Bogd Khan Palace, animal glue and egg white were identified as binding agents in architectural murals (Yang et al., 2010). In Europe, it was commonly employed in oil paintings and wall murals (Dallongeville et al., 2011; Johnson and Packard, 1971).In China, Neolithic findings indicate that humans boiled animal tissues to produce glue. (Lu, 2023). It was evident from Tang Dynasty paintings that animal glue was utilized to solidify inorganic pigments during that period (Zhang, 1963). In order for the mineral pigments to bond to the walls and work as a fixative for the murals' long-term preservation, a certain amount of animal glue was additionally combined during the painting process of the Mogao Grottoes of Dunhuang murals (Chen et al., 2005; Bomin et al., 2005; Shi, 1995). Animal glue was also used in the terra cotta warriors painting in the Qin Shihuang

* Corresponding author.
E-mail address: wq5457958101@126.com (Q. Wu).

https://doi.org/10.1016/j.jas.2025.106305
Received 19 December 2024; Received in revised form 16 June 2025; Accepted 18 June 2025
Available online 30 June 2025

Mausoleum (Bonaduce, 2008). Also animal glue has been found to be a bonding agent in Qingzhou's ceramic figurines and Jiayuguan's colored architectural paintings (Wang et al., 2009; Shuya et al., 2009; Wei et al., 2012). Notably, a 3500-year-old bovine glue was found adhering wooden canes in the Xiaohe Cemetery in Xinjiang (Yang, 2016). In northern China, dog glue was found to be a bonding agent in the murals of tombs from the 5th century (Fei et al., 2014).

However, the cultural heritage themselves contain relatively little of these animal glue components, which are often mixed with other inorganic materials and may also be contaminated by other inorganic and organic materials. The study of animal bonding materials is a quite challenging subject in the field of contemporary cultural heritage analysis due to the limitations of analysis and detection methods. Currently, there are three categories of conventional analysis and detection techniques: chemical, physical, and biological methods. Chemical methods involve the use of phenolphthalein to detect blood and Coomassie Brilliant Blue to detect proteins (Li et al., 2019).Although this method is quick and easy, they suffer from relatively high detection limits. Moreover, contamination can interfere with colorimetric results, leading to potential inaccuracies.Spectroscopy, chromatography, mass spectrometry, and capillary electrophoresis are among the frequently employed physical analysis techniques (Wang et al., 2011; Jurado-López and de Castro, 2004; Andreotti et al., 2006; Colombini et al., 2010). Although spectral chromatography and other techniques can identify the presence of animal collagen, they cannot determine the specific animal source due to the high structural similarity between protein molecules. Proteomic approaches enable species identification (Yang, 2016; Kuckova et al., 2013; Kaml et al., 2004), but they require a comprehensive biological mass spectrometry platform, which involves substantial initial investment and lengthy processing times. Additionally, it necessitates the creation of a database for samples of cultural heritage and the selection of the database's scope according to the historical details and geographic setting of the samples, which makes it challenging to quickly and easily identify species.

Enzyme-linked immunosorbent assay (ELISA) has been employed for biological analysis of cultural heritage materials (Kerkaert et al., 2010). However, this approach presents several challenges: aging cultural relics samples require particular antibodies to be screened, and a variety of antibodies must be developed before studies are conducted on cultural relics samples with unknown sample information. Extensive testing may deplete valuable cultural relic samples if material information cannot be determined. Structural changes in aged collagen proteins can cause cross-reactivity between different animal sources, potentially yielding inaccurate results. Furthermore, the method requires careful optimization to avoid false positives caused by non-specific binding (Palmieri et al., 2011). To overcome these limitations, DNA analysis has emerged as a more reliable approach for precise identification of animal glue sources in cultural artifacts.

In recent years, DNA barcoding technology has been developed as an innovative biological method for species identification (Cattaneo et al., 1991; Anava et al., 2020).This technique utilizes standardized, highly variable, easily amplifiable, and relatively short DNA segments that serve as species-specific genetic markers. In this study, In this study, we employed DNA barcoding to design universal primers targeting partial regions of the mitochondrial cytochrome *b* (Cyt *b*) gene. These primers were optimized for species commonly used in historical glue production, including pigs (*Sus scrofa*), cattle (*Bos taurus*), water buffalo (Bubalus bubalis), goats (*Capra hircus*), sheep (*Ovis aries*), camels (*Camelus bactrianus*), deer (*Cervus elaphus*), dogs (*Canis lupus* familiaris), horses (*Equus caballus*), and rabbits (*Oryctolagus cuniculus*). In order to identify the species source of animal glue in cultural heritage, the appropriate pieces were amplified and put through Sanger sequencing. This research establishes a novel biological detection method for identifying historical animal glue sources, offering significant advantages over traditional protein-based approaches.

2. Methods

1. Produce animal glue samples

1)Materials: Fresh animal hides (water buffaloes, pigs, yellow cattle, goats, sheep, camels, deer, dogs, horses, and rabbits; 50g each) were obtained from local abattoirs. All specimens underwent depilation and defatting using surgical scalpels prior to rinsing with deionized water.2) Thermal Extraction: The prepared hides were cut into 0.5 × 0.5 cm fragments. Fragments were transferred into a 1L glass beaker containing 500 mL distilled water. Hydrolysis was conducted using a digital hotplate stirrer (IKA® RCT Basic) at 100 °C with continuous magnetic stirring (300 rpm).Evaporative losses were compensated by periodic replenishment with preheated (100 °C) distilled water to maintain constant fluid volume.The process was terminated when viscoelastic thread formation occurred (6h), as determined by ASTM D1084 viscosity standards.3)Filtration:Use a sterile needle to perforate the filter paper (0.5 mm in diameter) to prevent the glue phase from blocking. Filter the colloidal suspension through filter paper.Desiccation Process.4)The filtrate was cast into polystyrene Petri dishes.Controlled drying was performed in an environmental chamber (Binder KBF-240) under:Temperature: 20 ± 0.5 °C, Relative humidity:45 ± 2 %.Final product was obtained after 48 h when moisture content reached 8.5 ± 0.3 %. (Xiaoxi et al., 2021).

2. Extraction of DNA from animal glue samples

All the following experimental processes were conducted in a specialized DNA laboratory, following the principle of sterility. PCR and post-PCR steps are carried out in separate laboratories and were run with blank. The extraction was done three times independently for each sample to ensure the security of the molecular result. According to the instructions of the DNA extraction kit: (FastPure® Blood/Cell/Tissue/Bacteria DNA Isolation Mini Kit:DC112) A 10 mg of the animal glue sample was transferred to a centrifuge tube. Subsequently, 200 μL of lysis buffer and 50 μL of 20 mg/mL protein K solution were sequentially added. The mixture was vortexed to homogeneity and incubated at 56 °C for 24 h. After incubation, 200 μL of extraction buffer was added, and the solution was inverted thoroughly before being incubated at 50 °C for 40 min. Following this, 200 μL of anhydrous ethanol was introduced, and the mixture was vigorously shaken for 30 s to ensure complete homogenization.The resulting solution was transferred to a silica membrane adsorption column and centrifuged at 12,000 rpm for 30 s, with the flow-through discarded. The column was then washed twice with 500 μL of elution buffer, each time centrifuging at 12,000 rpm for 30 s and discarding the effluent. A final centrifugation at 12,000 rpm for 2 min was performed to remove residual wash buffer. The column was air-dried at room temperature for 3 min to evaporate ethanol traces.For DNA elution, 50 μL of elution buffer was applied to the central region of the adsorption membrane, and the column was incubated at room temperature for 3 min. The DNA was then recovered by centrifugation at 12,000 rpm for 2 min, with the eluate collected as the final DNA solution.

Take 1 μL genomic DNA sample and detect it on the ultra micro spectrophotometer. By comparing the ratio of A260/A280 and the concentration of sample DNA, and combining the results of agarose gel electrophoresis of genomic DNA samples, the quality of extracted genomic DNA is evaluated.

3. Design of specific primers and DNA amplification

Download the cytb genome sequences of sheep: NC001941.1, goat: NC005044.2, cattle: NC006853.1, water buffalo: MK499431.1, Camel: NC009628.2, pig: NC000845.1, dog: NC002008.4, rabbit: NC001913.1, horse: NC001640.1, red deer: NC007704.2 from the Genbank database (Its number is shown in Table S1). Align the cytb gene sequences of five

animals, use Oligo.7 to design universal primers for the conserved regions shared by the five species, and select hairpin-free structures and dimers.The designed primer has a Tm value of 60 °C and the highest score.Due to the fragmentation of DNA in historical samples and processed simulated samples, the amplifiable fragments are relatively short (less than 200bp).However, there aren't enough mutation sites in segments smaller than 100 bp to differentiate between species. Therefore, the amplification length is set to 100-200bp.Design degenerate primers for the differences in several bases between different species sequences, but the number of degenerate codons should not exceed three. The designed universal primers will be preliminarily screened and validated for their universal characteristics through Primer BLAST of NCBI.

4. PCR amplification

The entire DNA extracted from the animal glue could be amplified using PCR using the primers already designed.Thermal Cycling was conducted in a 25 μL reaction mixture, consisting of 2.0 μL (50 ng) of genomic DNA, 1.0 μL (10 10 μmol/L)of each primer, 12.5 μL PCR MIX. PCR MIX contains deoxyribonucleotide triphosphates (dNTPs) 、Taq polymerase and Mgcl2.Add ddH_2O to 25 mL. PCR was conducted using the following temperature profile: initial denaturation at 95 °C for 5min, then 35 cycles of 60 Sec at 95 °C, 60Sec at 53 °C, and 45 Sec at 72 °C; and finally, an extension reaction of 5 min at 72 °C.Every sample was amplified with universal primers twice to check the reproducibility. PCR products were visualized on a 2 % agarose gel-electrophoresis for appropriate size checking.The amplified PCR bands were subsequently visualized in UV transilluminator for the amplified fragment size validation.

5. DNA sequencing and bioinformatics analyses

The PCR product was subjected to Sanger sequencing. Authenticity of sequences was determined by analyzing the reads obtained for each product and by considering at least three independent amplifications (independent PCRs) and three independent sequencing for each sample. The sequencing results were compared with the original sequences of the species using Jalview for sequence comparison, and the sequencing results were input into NCBI for online BLAST.

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn) homology comparison, to determine the species of the submitted sequences by analyzing the matching sequences with the highest similarity in the comparison results, and to identify the species.

6. Exploration of the detection limit of animal glue in simulated mortar samples
   1) Different ratios of animal glue were used to create samples of simulated cement:Lime (calcium oxide) and water were combined to create a slurry mixture, which was then aged for seven days in a cold indoor environment to replicate the inorganic mortar substrate.Adding water while using lime turns it into hydrated lime.The chemical reaction equation is: $CaO + H_2O=Ca(OH)_2$.After an aging reaction, calcium carbonate is created when hydrated lime reacts with atmospheric carbon dioxide.The chemical reaction equation is: $Ca(OH)_2+CO_2=CaCO_3\downarrow+H_2O$. Both calcium hydroxide and calcium carbonate are currently present in the simulated mortar sample.At this stage, take 10g of the mortar sample and mix it uniformly by adding varying weights of cowhide glue solution.
   2) Extract DNA from simulated samples:Calcium carbonate and calcium hydroxide must be eliminated from the sample using acetic acid prior to DNA extraction since their presence in simulated mortar samples may impact the results of subsequent DNA extraction.Through experiments, calcium hydroxide and carbonate were extracted from simulated samples using a 3M acetic acid solution. The chemical reaction equation is:

$$CaCO_3 + 2CH_3COOH = Ca(CH3COO)_2 + H_2O + CO_2\uparrow$$

$$2CH_3COOH + Ca\ (OH)_2 = (CH_3COO)_2Ca + 2H_2O。$$

Put 100 μ L of a 3M acetic acid solution into a test tube containing 10 mg of mortar sample. To eliminate impurities, centrifuge the simulated mortar sample at 12,000 rpm for 5 min after reacting calcium hydroxide and carbonate. Use 3M Tris HCl (pH12) to bring the resultant solution's pH to 7. Using the above experimental steps, DNA extraction and amplification were performed on simulated mortar samples containing different weights of cattle glue.

7. Animal glue species identification in mortar cultural heritage

Historical samples (QSW-1、JQTWM-1、DCD-1、Shaanxi-2、Shaanxi-3、LZX1、LZX-16、LZX-18) must be preprocessed to remove DNA contamination from microbes, other animals, and plants before DNA can be extracted.The sample pretreatment work is carried out in a sterile,ultra-clean bench.

1) Use 70 % alcohol to remove dust and contaminants from the mortar sample's surface. 2) Use a UV lamp to illuminate the mortar sample for 15 min 3) Take a sample from the inner layer of the mortar sample that hasn't come into contact with the outside world after removing the outer layer. 4) Put 100 μ L of a 3M acetic acid solution into a test tube containing 10 mg of mortar sample. To eliminate impurities, centrifuge the simulated mortar sample at 12,000 rpm for 5 min after reacting calcium hydroxide and carbonate. 5) Use 3M Tris HCl (pH12) to bring the resultant solution's pH to 7. Perform DNA extraction, amplification, and sequencing using the method mentioned in step 3.4.5.

3. Results

1. Appearance of animal glue samples

Pigs, cattle, water buffaloes, goats, sheep, camels, deer, dogs, horses, and rabbits were used in this study to make animal glue, which turned out to be a light brown or white viscous liquid. Dip a glass rod into the adhesive solution, and there will be viscosity when the adhesive drops. The appearance of each species of adhesive is shown in the Fig. 1.

2. DNA extraction, amplification and detection results of animal glue

Table 1 shows the extracted animal glues' DNA concentrations and A260/280. The universal primers designed in this study is:

Cytbf: 5′-GACAAAATYCCATTYCACCC-3′,

Cytbr:5′-GGTGTAGTTRTCTGGGTCTCC-3'.

DNA fromglues derived from pigs, cattle, water buffalo, goats, sheep, camels, deer, dogs, horses, and rabbits is amplified using universal primers.The agarose gel electrophoresis results are displayed in the Fig. 2, and the blank control for amplification is deionized water.The results of gel electrophoresis showed that ten kinds of animal glue could amplify clear target bands.Each sample's bidirectional DNA sequencing peaks were subjected to error base correction and upstream/downstream result concatenation using Snapgene software. The sequencing results were submitted to GenBank for Blast analysis. The BLAST search results are shown in the table, and each animal can be successfully identified.

The sequencing results of the animal glues were aligned with standard reference sequences, as shown in Figure FS1. Several mutations were observed in the sequencing data, including T-to-C, C-to-A, C-to-T, and T-to-A substitutions (highlighted in green boxes).This phenomenon could be attributed to:Genetic mutations in the animals used to produce

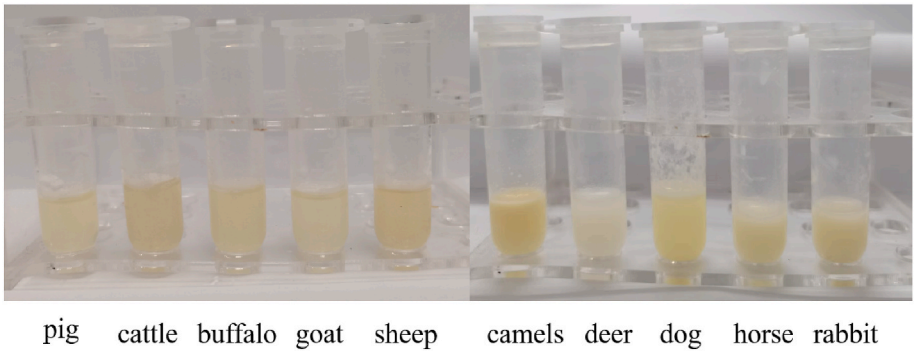

Fig. 1. Appearance pictures of ten different animal glues.

Table 1
Animal glue detection results in simulated mortar samples.

Weight of mortar (mg)	Weight of cattle glue (mg)	Ratio	DNA concentration (ng/uL)	A260/280
10	2	5:1	98.53	1.52
10	1	10:1	74.59	1.50
10	0.1	100:1	41.12	1.45
10	0.05	200:1	34.26	1.63
10	0.02	500:1	23.59	1.42
10	0.01	1000:1	10.12	1.40
10	0.005	2000:1	–	–

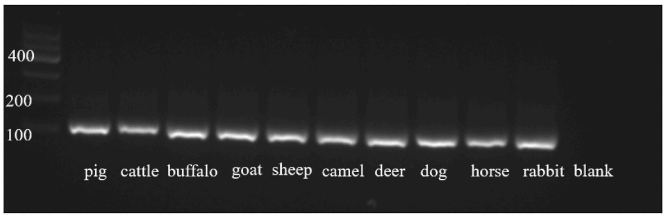

Fig. 2. Gel electrophoresis of PCR amplification results of ten different animal glue DNA.

the glue, DNA alterations during the simulated sample preparation process, or Sequencing errors.However, these sporadic nucleotide substitutions did not affect species identification and do not compromise the reliability of the analytical technique developed in this study.

The software MEGA was employed to calculate intra- and interspecific genetic distances based on the K2P model, with the complete deletion option for gaps/missing data in sequence alignments (i.e., sites containing insertions or deletions were excluded from analysis). The genetic distances are summarized in Table S2.The interspecific genetic distances among the ten animal species ranged from 0.079 to 0.317, while intraspecific distances ranged from 0.000 to 0.087, with an average interspecific distance of 0.197. The intraspecific distances were significantly lower than interspecific distances, consistent with the criterion that interspecific divergence should exceed intraspecific divergence by at least 10-fold, allowing reliable species identification. Since the intraspecific variation in each sample was markedly lower than interspecific divergence, the species of all animal glues samples could be successfully determined.

The ML tree (1000 bootstrap replicates), constructed using reference sequences (Table S1) and animal glue samples, demonstrated that all samples clustered reliably with their respective species (Fig. S2), validating species-level resolution of the method.

Based on the comprehensive evaluation of BLAST, genetic distance calculation, and maximum likelihood tree construction results, all ten animal glues can be successfully identified at the species level.

3. Test results of animal glue with different proportions in simulated mortar samples

Mix simulated mortar and cattle glue of different qualities in different proportions, as shown in the table.The table displays the DNA concentrations that were extracted from samples that had different proportions.The DNA that was extracted performed gel electrophoresis after being amplified. It is shown that DNA can still be extracted and amplified at a 1000:1 ratio of mortar to glue. Fig. 3 displays the gel electrophoresis results of the amplified products, which exhibit bands that are both clear and the right size. DNA extraction is unsuccessful when the ratio is 2000:1. Show that this method can identify at least 1000:1.

4. Animal glue species identification in mortar cultural heritage

Sample number	Animal glue	Amount used (mg)	DNA concentration (ng/uL)	A260/280	BLAST Result	Query cover	E-value	Per. Identity	Top score GenBank accession number
1	sheep	10	98.53	1.52	*Ovis aries*	100 %	7e-52	98.39 %	OR459712.1
2	goat	10	74.59	1.50	*Capra hircus*	100 %	1e-49	96.85 %	MF503654.1
3	cattle	10	41.12	1.45	*Bos taurus*	100 %	5e-54	99.21 %	MZ901474.1
4	water buffalo	10	84.26	1.63	Bubalus bubalis	100 %	2e-51	97.62 %	MT182582.1
5	camel	10	83.59	1.42	*Camelus bactrianus*	99 %	1e-49	96.80 %	MH109896.1
6	pig	10	90.12	1.40	*Sus scrofa*	100 %	7e-52	97.62 %	KU664548.1
7	rabbit	10	82.89	1.59	*Oryctolagus cuniculus*	100 %	3e-50	96.83 %	AJ539456.1
8	dog	10	107.01	1.55	*Canis lupus* familiaris	100 %	1e-55	100.00 %	OQ340890.1
9	horse	10	54.55	1.45	*Equus caballus*	100 %	6e-53	98.41 %	KT221842.1
10	red deer	10	100.12	1.59	*Cervus elaphus*	100 %	8e-45	93.65 %	MT266654.1

DNA concentration and purity of samples and results of BLAST analysis.

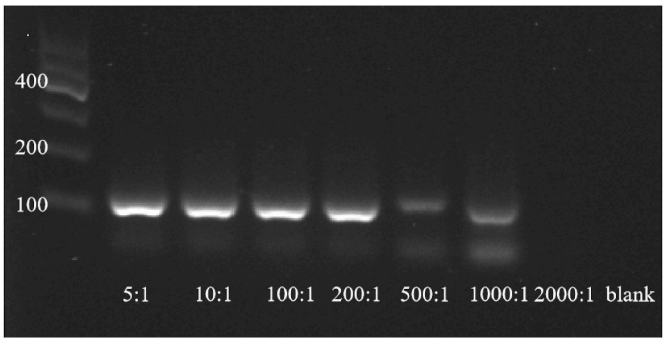

Fig. 3. Amplified gel electrophoresis results of DNA extracted from different proportions of animal glue.

The samples for this study were three Neolithic mortar samples from Yulin City, Shaanxi Province, three mortar samples from the Forbidden City in Beijing, and three mortar samples from the Confucian Temple in Shandong Province. The geographical distribution of the eight samples is shown in Fig. 4.The detailed information of mortar cultural heritages was shown in Table 2. The DNA extraction and identification results are shown in the table, and according to the BLAST results, the species can be accurately identified. The sequences obtained from this experiment have been uploaded to the NCBI database, and the login numbers are shown in the table.

The sequencing results of the mortar cultural relics were compared with the standard reference sequences of *Bos taurus* (cattle), *Sus scrofa* (pig), and Bubalus bubalis (water buffalo), as shown in Fig. S3.Samples QSW-1, JQTWM-1, DCD-1, Shaanxi-2, Shaanxi-3, LZX-1, LZX-16, and LZX-18 exhibited C-to-T mutations (highlighted in green boxes) in their sequencing results.Additionally, the historical samples in this study exhibited C-to-T mutations—a feature rarely observed or entirely absent in the modern animal glue samples. This phenomenon aligns with

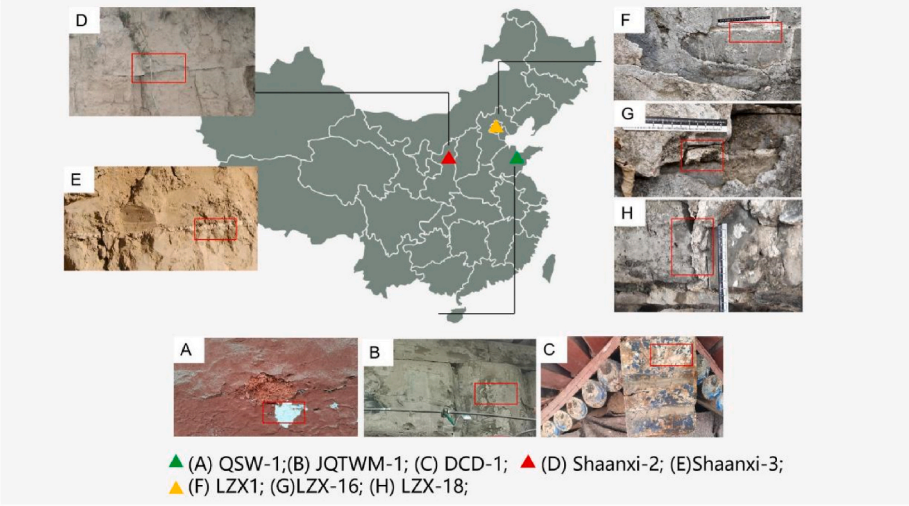

Fig. 4. The geographical location of mortar samples from eight cultural heritages.

Table 2
Sample data and sequencing result.

Sample number	Sample name	Locality	Amount used (mg)	DNA concentration (ng/uL) and A260/A280	Animal species (Latin name)	Top score GenBank accession number	E-value	Per.Identity	Query cover	GenBank accession number
A	QSW–1	Temple of Confucius, Shandong, China	100 mg	40.25 1.45	water buffalo	OR766454.1	3e-51	97.62 %	100 %	PQ753935
B	JQTWM-1	Temple of Confucius, Shandong, China	100 mg	35.33 1.50	water buffalo	OR766454.1	3e-51	97.62 %	100 %	PQ753936
C	DCD-1	Temple of Confucius, Shandong, China	100 mg	30.49 1.49	Pig	OQ127625.1	1e-48	96.03 %	100 %	PQ753937
D	Shaanxi-2	Yulin, Shaanxi, China	100 mg	20.10 1.52	cattle	FJ530479.1	5e-54	99.21 %	100 %	PQ753938
E	Shaanxi-3	Yulin, Shaanxi, China	100 mg	24.22 1.55	cattle	MT576713.1	1e-55	100 %	100 %	PQ753939
F	LZX1	The Imperial Palace, Beijing, China	100 mg	31.22 1.44	water buffalo	MK499428.1	9e-51	97.62 %	100 %	PQ753940
G	LZX-16	The Imperial Palace, Beijing, China	100 mg	30.25 1.56	water buffalo	MK499428.1	5e-54	99.21 %	100 %	PQ753941
H	LZX-18	The Imperial Palace, Beijing, China	100 mg	29.33 1.55	water buffalo	MK499428.1	8e-45	93.65 %	100 %	PQ753942

characteristic ancient DNA damage patterns, where post-mortem nucleotide misincorporation typically manifests as C-to-T or G-to-A substitutions in sequence fragments. Such damage profiles further corroborate the authenticity of the historical samples.

The software MEGA was employed to calculate intra- and interspecific genetic distances based on the K2P model, with the complete deletion option for gaps/missing data in sequence alignments (i.e., sites containing insertions or deletions were excluded from analysis). The genetic distances are summarized in Table S3. Since the intraspecific variation in each sample was markedly lower than interspecific divergence, the species of all animal glues samples could be successfully determined.

The ML tree (1000 bootstrap replicates), constructed using reference sequences (Table S1) and historical samples.The phylogenetic reconstruction results are presented in Fig. S4. The samples QSW-1 and JQTWM-1 from Qufu, Shandong Province, along with three samples from the Forbidden City (LZX1, LZX-16, and LZX-18), clustered with the water buffalo (Bubalus bubalis) reference sequence. Sample DCD-1 from Qufu, Shandong was assigned to the pig (*Sus scrofa*) reference sequence. Meanwhile, the two mortar samples from Yulin City, Shaanxi Province (Shaanxi-2 and Shaanxi-3) grouped with the cattle (*Bos taurus*) reference sequence.

Based on the comprehensive evaluation of BLAST, genetic distance calculation, and maximum likelihood tree construction results, 8 historical samples can be successfully identified at the species level.

4. Discussion

It is challenging to identify these animal cement components by chemical and physical testing because they are mixed with other inorganic minerals and have very little amount in the cultural heritage.The methodology developed in this study demonstrates superior sensitivity, with a detection limit for animal glue that is markedly lower than that achievable through traditional protein analysis in mortar samples using chemical approaches.The robustness of our method is evidenced by its successful application to historical mortar samples spanning different chronological periods and geographical locations, including:Neolithic period artifacts from Yulin, Shaanxi Province; Ming and Qing Dynasty materials from the Forbidden City in Beijing; Qing Dynasty samples from the Confucian Temple in Qufu, Shandong Province.These results confirm that our analytical approach is not constrained by either the age of the sample or its geographical provenance, representing a significant advancement in the characterization of organic binding media in architectural heritage.

Our analysis revealed the presence of cattle-derived animal glue in Neolithic mortar samples from Yulin City, Shaanxi Province. This finding provides important insights into early human utilization of animal byproducts. In prehistoric societies, after consuming animal meat, people systematically repurposed various tissues, including skin and bones. Supporting evidence comes from zooarchaeological studies documenting numerous cattle skeletal remains in the Yulin region where the mortar samples were collected (Hu et al., 2008; Yu et al., 2022). The identification of cattle glue rather than wild animal derivatives in Neolithic masonry suggests two significant developments: the advancement of livestock domestication, and the systematic exploration of non-alimentary applications for animal resources. Cattle glue is durable and has a nice viscosity. It was used for an extended period of time in ancient China.Historical applications of cattle glue include:As an adhesive for bone engravings at Xiaohe Cemetery (Luobupo, Xinjiang) during the Shang and Zhou periods (Yang, 2016). As a binding agent for wooden bows and arrows discovered at Yanghai Cemetery in Turpan.As a mortar additive, as demonstrated by our Neolithic samples.This study establishes that cattle glue applications extended beyond simple adhesives to include construction materials as early as the Neolithic period (Palidanmu, 2020).The use of cattle glue as a mortar additive represents a significant technological innovation, enhancing the performance of early building materials.

The production and utilization of animal glue has undergone continuous development throughout Chinese history. The Northern Wei Dynasty's agricultural classic book "Qi Min Yao Shu" (Essential Techniques for the Welfare of the People) has comprehensive records of the raw materials and cooking techniques for glue, demonstrating that 1400 years ago, China had a developed and sophisticated theoretical system and technology for producing animal glue (Jia, 2015). By the Ming and Qing dynasties, animal glue manufacturing had reached an advanced stage of development. The widespread practice of cattle husbandry in China made bovine-derived glues particularly prevalent.The book "Code of Conduct for Qin Ding Qing Dynasty" records that "water glue is mainly supplied by Shandong, Henan, Anhui, Jiangxi, Fujian, Zhejiang, Hubei, Hunan, and Guangdong," indicating that these provinces are the main suppliers of glue (The Imperial Code of the, 1986).Our analysis of mortar samples from the Forbidden City confirmed the use of water buffalo glue during this period, consistent with the production methods described in "Qi min Yao shu".The historical texts reveal careful consideration in the selection of raw materials. As noted in Qimin Yaoshu: "While donkeys and horses produce greater quantities of glue due to their thinner hides, the production costs are twice as high" (Jia, 2015). This economic rationale, combined with the ready availability of bovine materials (including hide, bone, and tendon), made water buffalo the preferred source for architectural adhesives, particularly where large quantities of binder were required for mortar production.Fish bladder glue, which is used to join wooden objects, is another popular kind of animal glue in the Forbidden City. However, compared to cattle or water buffalo glue, fish meal glue has a longer production time, more complex manufacturing methods, is more susceptible to bacterial and fungal contamination, and is more prone to failure, decomposition, and spoilage (Wang et al., 2024).These factors ultimately precluded its use as a binder in construction mortars, despite its excellent adhesive properties for woodworking applications.

Analysis of Qing Dynasty mortar samples from the Confucian Temple in Shandong Province revealed the use of porcine-derived glue, indicating that historical builders did not adhere to strict specifications regarding animal glue types for mortar production.It is supposed that the selection of animal to produce glue may be based on the availability of animals in the area, as well as other operational and material considerations.For instance, because its raw materials are found around the coast and in places with extensive river networks, fish glue is made extensively in coastal regions like Jiangsu, Zhejiang, Fujian, and Shanghai. Northeastern China, central Inner Mongolia, Ningxia, southeast Xizang, northern Qinghai, Gansu, Shaanxi, western Sichuan, and other deer distribution zones in China are the primary locations for deer glue (Liu et al., 2023).Therefore, the identification of animal glue can support the advancement of agriculture and animal husbandry during that age, corroboration of documentary records in ancient texts.The regional variation in glue production materials reflects both the practical adaptation to local resources and the accumulated empirical knowledge of traditional building material optimization.

5. Conclusion

This study demonstrates that ten common species in animal glue can be detected using the DNA barcoding method and a pair of universal primers for identification all of them at once experiment. Compared to other physical, chemical, and biological procedures, the DNA barcoding technique used in this study can be much more accurate, even to the species level. Impurities in the sample have no effect on the results and the detection limit is 1000:1. Samples from various time periods and places can yield accurate identification results. The authenticity of the materials documented in ancient documents is further confirmed by our results. The fact that the source species of animal glue are compatible with typical local animals indicates that the species chosen for animal glue production must be in line with the distribution of local species.

This study provides a suitable detection method for the species source detection of cultural heritages.

CRediT authorship contribution statement

Qian Wu: Writing – original draft. **Bingjian Zhang:** Supervision. **Qiong Zhang:** Resources. **Mingzhi Ma:** Resources. **Yulan Hu:** Writing – review & editing.

Data availability

The data that support the findings of this study are included as part of the paper and supplementary materials and GenBank database.

Funding

This research was funded by [National Key R&D Program of China] grant number [No. 2022YFF0903800].

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2025.106305.

References

Anava, S., Neuhof, M., Gingold, H., et al., 2020. Illuminating genetic mysteries of the dead sea scrolls. Cell 181 (6), 1218–1231.
Andreotti, A., Bonaduce, I., Colombini, M.P., et al., 2006. Combined GC/MS analytical procedure for the characterization of glycerolipid, waxy, resinous, and proteinaceous materials in a unique paint microsample. Anal. Chem. 78 (13), 4490–4500.
Bomin, Su, Beizhefu, Zhen, Zhide, Hu, et al., 2005. HPLC analysis of adhesive materials for Kizil Grottoes mural paintings. Dunhuang Studies (4), 57–61+116.
Bonaduce, Ilaria, 2008. The binding media of the polychromy of Qin Shihuang's Terracotta Army. J. Cult. Herit. 9 (1), 103–108.
Cattaneo, C., Gelsthorpe, K., Phillips, P., et al., 1991. Identification of ancient blood and tissue–ELISA and DNA analysis. Antiquity 65 (249), 878–881.
Chen, Guangde, Zhang, Li, Zuixiong, Li, et al., 2005. Determination of proteins in Dunhuang mural pigment binding materials by spectrophotometry. J. Northwest Normal Univ. (Nat. Sci.) 1 (01.016), 47–50.2005.
Colombini, M.P., Andreotti, A., Bonaduce, I., et al., 2010. Analytical strategies for characterizing organic paint media using gas chromatography/mass spectrometry. Accounts Chem. Res. 43 (6), 715–727.
Dallongeville, Sophie, Koperska, Monika, Garnier, Nicolas, et al., 2011. Dentification of animal glue species in artworks using proteomics: application to a 18th century gilt sample. Anal. Chem. 83 (24), 9431–9437.
Fang, Shi Qiang, 2014. The identification of organic additives in traditional lime mortar. J. Cult. Herit. 15 (2), 144–150.
Fei, Yan, Qinya, Ge, Qiang, Li, et al., 2014. Preliminary study on dog collagen in adhesive materials of ancient tomb murals using enzyme linked immunosorbent assay (ELISA). Cultural Relics Protection and Archaeological Science 26 (1), 71–75.
Hu, Songmei, Zhang, Pengcheng, Yuan, Ming, 2008. Research on animal remnants at Yulin Huoshiliang Site. Anthropol. J. (3), 232–248.
Jia, Sixie, 2015. Qi Min Yao Shu [M]. Zhonghua Book Company, Beijing.
Johnson, M., Packard, E., 1971. Methods used for the identification of binding media in Italian paintings of the fifteenth and sixteenth centuries. Stud. Conserv. 16 (4), 145–164.
Jurado-López, A., de Castro, M.D.L., 2004. Use of near infrared spectroscopy in a study of binding media used in paintings. Anal. Bioanal. Chem. 380, 706–711.
Kaml, I., Vcelakova, K., Kenndler, E., 2004. Characterisation and identification of proteinaceous binding media (animal glues) from their amino acid profile by capillary zone electrophoresis. J. Separ. Sci. 27 (3), 161–166.
Kerkaert, B., Mestdagh, F., De Meulenaer, B., 2010. Detection of hen's egg white lysozyme in food: comparison between a sensitive HPLC and a commercial ELISA method. Food Chem. 120 (2), 580–584.
Kuckova, S., Sandu, I.C.A., Crhova, M., et al., 2013. Protein identification and localization using mass spectrometry and staining tests in cross-sections of polychrome samples. J. Cult. Herit. 14 (1), 31–37.
Li, Jiajia, Zhang, Bingjian, 2019. Why ancient Chinese people like to use organic–inorganic composite mortars?—Application history and reasons of organic–inorganic mortars in ancient Chinese buildings. J. Archaeol. Method Theor 26, 502–536.
Liu, Shuang, Yuehua, Zhao, 2023. Comprehensive study on commonly used adhesives in ancient China. Front. Archaeol. Res. (2), 374–390.
Lu, Hui, 2023. Organic additives to Neolithic white lime surfaces in Yulin, Shaanxi, China. J. Archaeol. Sci. 158, 105841.
Palidanmu, 2020. Shading research on wooden objects excavated from Turpan Yanghai cemetery and their uses. Turpan Stud. Res. (1), 49–60+154.
Palmieri, M., Vagnini, M., Pitzurra, L., et al., 2011. Development of an analytical protocol for a fast, sensitive and specific protein recognition in paintings by enzyme-linked immunosorbent assay (ELISA). Anal. Bioanal. Chem. 399, 3011–3023.
Shi, Li, 1995. Quantitative analysis of bonding materials in Dunhuang murals. Dunhuang Studies (3), 29–46.
Shuya, Wei, Ma, Qinglin, Scjreiner, M., 2009. Research on adhesive materials for colored pottery figurines in Xiangshan Han tombs, Qingzhou, Shandong. Relics and Museolgy 6, 71–78.
Sweatt, H.B., 1946. The properties of animal glue. J. Chem. Educ. 23 (4), 192.
The Imperial Code of the Qing Dynasty During the Qianlong Period[M], 1986. The Commercial Press of Taiwan.
Wang, Liqin, Yang, Lu, Cao, Xueyun, et al., 2009. Analysis and Study of Bonding Materials in Cultural Relic Pigments [C]. Academic Annual Meeting of China Association for the Protection of Cultural Relics, pp. 321–325.
Wang, Liqin, Yang, Lu, Zhou, Wenhui, et al., 2011. Comparison and evaluation of several protein determination methods in mural and architectural painted cultural relics. Cultural Relics Protection and Archaeological Science 23 (2), 59–63.
Wang, Dan, Gong, Tianshu, Wang, Xinyi, etc, A., 2024. Study on the paper lining of gold and silver thread in qing dynasty silk weaving in the Palace Museum collection. China Paper Industry 43 (10), 19–29.
Wei, S., Ma, Q., Schreiner, M., 2012. Scientific investigation of the paint and adhesive materials used in the Western Han dynasty polychromy terracotta army, Qingzhou, China. J. Archaeol. Sci. 39 (5), 1628–1633.
Xiaoxi, Li, Qiang, Luo, Bowen, Jing, et al., 2021. Preliminary study on simulation of ancient rubber making process. Contemp. Chem. Res. (14), 137–138.
Yang, Yimin, 2016. The application of ancient protein analysis in archaeology. J. Zhengzhou Univ. (Eng. Sci.) 49 (4), 102–105.
Yang, Lu, Wang, Liqin, Huang, Jianhua, et al., 2010. Amino acid analysis method for studying the types of adhesive materials used in the architectural color paintings of Bogdakhan Palace in Mongolia. Anal. Chem. 38 (7), 1044–1047.
Yu, C., Zhao, H., Hu, S., et al., 2022. A passion for beef: Post-domestication changes in cattle body size in China from the Late Neolithic to the Late Bronze Age. Front. Ecol. Evol. 10, 949860.
Zadrożna, I., Połeć-Pawlak, K., Głuch, I., et al., 2003. Old master paintings–a fruitful field of activity for analysts: targets, methods, outlook. J. Separ. Sci. 26 (11), 996–1004.
Zhang, Yanyuan, 1963. Records of Famous Paintings of Various Dynasties [M]. People's Fine Arts Publishing House, Beijing.