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Animal species identification utilising DNAs extracted from traditionally manufactured gelatin (*Wanikawa*)

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Abstract

Gelatin, sourced from collagen, is an acid-, alkali- or enzymatically hydrolysed product obtained from animal skins and bones. Gelatin has been widely used for the manufacture of various cultural objects, e.g. as a water-soluble binder for dissolving pigments, and as a glue for musical instruments and traditional crafts along with human history. The identification of animal species in gelatin, hence, could provide a critical clue for understanding human history including lifestyles, the culture and the technologies. However, there has been no valid method established to date for identifying the animal species from traditional gelatins. We herein report that the nucleic acids contents (dsDNA, ssDNA and miRNA) from commercially-available gelatins manufactured according to classical procedures (*wanikawa*) exhibited much higher (about 10 times) than those from modern gelatins made through an industrialised process (*yonikawa*), suggesting that DNA analysis using the gelatins from cultural assets could be substantially feasible. Moreover, targeting not only commercially available *niwaka* but also *Ukiyo-e*, Japanese classical art manufactured through wood-block printings, we here illustrate partial successes in the animal species identification coupled with DNA barcoding technique, hopefully paving the way for scientifically more reliable animal species identifications of archaeological specimens made with a gelatin component.

Keywords: Gelatin, DNA, DNA extraction, *Nikawa*, *Wanikawa*, *Yonikawa*

Introduction

Glue, or gelatin (*nikawa*) is a crude protein fabricated from the animal bones or skins of cow, pig, rabbit and deer, and squamosa of fish etc., of which the main component is collagen protein. *Nikawa* has been used for a variety of purposes including adhesives for art crafts and architectures, ink cakes and pigment fixers [1, 2]. *Nikawa* with higher purity is called gelatin, historically utilised for foods, pharmaceuticals and photography as a fixing reagent for a long period [1, 2]. The history of *nikawa* is deeply rooted. For instance, *nikawa* was used as adhesive bonds for manufacturing artistically crafted products from 4000 B.C. and 3000 B.C. in China and Egypt respectively [1, 2]. In Japan, *nikawa* began to be utilised for adhesives and stickings from the seventh century, and today is still an indispensable tool for the restoration of cultural objects and fixing pigments [2]. In particular, *nikawa* plays vital roles in repairing cultural artefacts because modern and industrialised adhesives such as epoxies and cyanoacrylates might cause detrimental effects on cultural assets such as denaturation and discolouration [2].

In Japan, *wanikawa* represents *nikawa* materials manufactured through traditional procedures comprising unhairing, warm water extraction and concentration of collagen fibrils, filtration (optional) and solidification, followed by air-drying at ambient conditions [1, 2]. As the

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etymology of "*nikawa*" can be traced back to the ancient Japanese word, "boiled skin", the production of traditional *wanikawa* is thus characterised by "boiling" animal skins to concentrate collagen proteins. In contrast, *yonikawa* has been produced through modernised industrial processes including harsh purification steps like vacuum extraction and ion exchange, thus resulting in largely different collagen purities between *wanikawa* and *yonikawa* [1, 2]. Whereas the extraction and purification processes of traditional gelatins are not fully clear, the present production method of gelatin is largely classified into acidic method (Type A gelatin) and alkaline method (Type B gelatin) for solubilising collagen proteins [3] (Nippi Inc., personal communication). Acidic method is preferred for gelatins with sparse collagen tissue (e.g. pig skins, fish skins and scales) so that collagen fibrils can be easily solubilised. This method is characterized by acid-soaking after washing with water. For example, pig skin is soaked in 1–5% (v/v) sulphuric acid or hydrochloric acid for about 10–30 h. On the other hand, if the raw materials are bovine bones, bovine hides or pig bones which have a strong collagen structure, alkaline method is preferred. Water-washed materials are soaked in 1–5% (w/v) lime water for as long as 1–2 months, thus facilitating solubilisation of the fibrils by partial hydrolysis of collagen proteins. One of the great differences between traditional and industrialised methods would be that the modern method, both in Type A or Type B gelatins, includes ion exchange process for removing ionic substances, achieving higher purities of collagen proteins than those through classical procedures.

Identifying the animal species utilised for classically-manufactured *nikawa* (*wanikawa*) is thus expected to provide insight into ancient civilisations, including historical technologies in architecture and handcrafts, eating habits, cultural exchange and residential environment. However, there appears to be no consistent methodology for the species identification of *nikawa* utilised for cultural properties in conservation science and archaeological area. A proteomic approach called ZooMS (Zooarchaeology by Mass Spectrometry) has been developed for identifying animal species tracing proteins such as collagen [4, 5] and β-lactoglobulin [6] as intrinsic biomarkers respectively (reviewed in [7]). Apart from archaeology-related areas, species identifications through DNAs eluted from gelatins have been reported, for example, in pharmaceuticals targeting the donkey cytochrome b gene [8] and the donkey cytochrome c gene [9], as well as in food sciences, especially for halal authentication methods [10–13]. For halal authentication, real-time PCR method appears to be broadly applied since a rapid and simple detection platform is required for routine examination. Despite the practical convenience as a detection system, since real-time PCR sometimes gives false-positive results, careful construction of the control experiments ought to be taken [14]. Mass spectrometric approaches have been also employed for the source authentication [15–17]. However, because collagen proteins might be degraded in archaeological contexts, and collagens are known to be highly modified in general [18], it is assumed to be difficult to perform animal species identification relying on mass spectrometry-related techniques, which is likely to be greatly dependent upon how the samples have been stored. On top of the mass spectrometric approach, immunochemical methods including ELISA have been also employed for this purpose as has been reviewed in [13]. In spite of the similarities of the primary structures of collagen amongst animal species, immunological systems for the species identification with securing the specificities have been explored [10]. On the other hand, DNA-based approaches for animal identification have been extensively exploited [19–21], enabling the identifications not only of the animal species, but also of the animal strain or the origin through genotyping [22], thus providing extra data that may be useful for informing on cultural history. Although it has been broadly discussed which technique could be more advantageous for archaeological research [23], however, to our knowledge, DNAs contained in the archaeological *nikawa* (*wanikawa*) samples have not yet been studied.

In this report, molecular analysis using commercially available *nikawa* samples demonstrates herein that DNA contents per a weight of *wanikawa* are much higher than those in *yonikawa* through fluorescent quantification. We further attempted species identification by utilising extracted DNAs from *wanikawa* through DNA barcoding, resulting in partial identification. In this exemplification, not only to avoid false-positive results, but also to realise sequential analysis like Sanger and amplicon sequencing, we employed classical procedures including PCR with species-specific primers followed by agarose electrophoresis. Amplified fragments may be also utilised for nested-PCR to improve sensitivity and specificity. Also, to test the versatility of this DNA barcoding approach, animal species identification using *Ukiyo-e* manufactured between Edo and Meiji periods (~150 years ago) was performed, resulting in successful identification of the species utilised for fabricating the *nikawa*. We hope that the data exemplified herein could contribute to the further establishments of molecular biological approaches identifying archaeological samples.

Materials and methods

Gelatin samples

Gelatin (*nikawa*) samples were purchased on April, 2022, from: Kremer Pigmente Co., Ltd., Germany, Sankichi Co.,

Ltd., Japan, PAReT Co, Ltd., Japan, Kissho Co, Ltd., Japan (gelatins for painting); Morinaga Co., Ltd., Japan and House Foods Co., Ltd., Japan (gelatins for cooking); Nippi Inc., Japan (standard *yonikawa* with identified sources). Specifications for each gelatin samples are listed in Table.1. Gelatins according to the classical method (gelatins #1 to #11) are generally termed as *wanikawa*, whose purification procedures are not so intense as industrialised *yonikawa* (gelatins #12 and #16) as described above. Whilst gelatin #17 is for painting provided as a solution, the process of the manufacture (*wanikawa* or *yonikawa*) was unclear. All the gelatin samples were stored in ambient condition and avoiding direct sunlight. During the experiments, gelatin samples were carefully handled so as to avoid contaminations from the experiment practitioners, e.g. appropriately using disposable gloves and clean labwares.

de novo fluorometric DNA quantification

Each gelatin sample was weighed (~0.1 g) and recorded, and distilled water was added up to 1.0 mL, followed by heating at 70 °C with vortexing until the gelatin samples fully dissolved. Nucleic acid quantifications were performed by Qubit 4 Fluorometer (Thermofisher Scientific, MA). 10 µL of gelatin solution was mixed with 190 µL of each fluorescent solution (Qubit™ dsDNA HS Assay Kit (#32,851, Thermofisher Scientific), Qubit™ ssDNA Assay Kit (#Q10212, Thermofisher Scientific), and Qubit™ microRNA Assay Kit (#Q32880, Thermofisher Scientific)), and quantified as illustrated in [24]. These dyes are shown to specifically bind to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) and mircoRNA (miRNA) respectively to give rise to characteristic fluorescence [25]. Each quantification procedure was carried out in triplicate (n = 3).

DNA extraction from the gelatin samples

The homogeneous gelatin solutions as prepared above were subjected to DNA extraction according to the manufacturer's instructions (Analytik Jena: PME Gelatin DNA Kit, #845-IR-0007050). Briefly, distilled water was added to 0.1 g of each gelatin sample to make 10% (w/v) gelatin solution and dissolved at 70 °C, overnight. The gelatin solution was then subjected to Proteinase K digestion, followed by polymer-based DNA purification procedures. This DNA crude extract was loaded onto silica spin columns for the final purification to yield purified DNA solution suitable for molecular experiments including PCR.

Species identification by PCR or DNA sequencing

In this study, we principally followed species identification through DNA barcoding system as disclosed in the literature (Additional file 1: Table S1) [26–30]. Briefly, DNA was amplified with sets of primers targeting animal mitochondrial *COI* (cytochrome c oxidase subunit I) gene [26], cattle mitochondrial genome (region extending position 8108 to 8378 including *trnK-UUU* ($tRNA^{Lys}$), *ATP8* (ATP synthase F_0 subunit 8) and *ATP6* (ATP synthase F_0 subunit 6) genes) [27, 28], sheep cytochrome

Table 1 Gelatin samples analysed in this study

Sample No.	Specification	Origin	Code
1	Bone glue, pearls	Cattle	Kremer Pigmente, Germany #63000
2	Hide glue	unspecified	Kremer Pigmente, Germany #63020
3	Rabbit skin glue	Rabbit	Kremer Pigmente, Germany #63025
4	Rabbit skin glue	Rabbit	Kremer Pigmente, Germany #63028
5	Technical gelatin	Cattle	Kremer Pigmente, Germany #63045
6	Bookbinding glue	Cattle	Kremer Pigmente, Germany #63060
7	Parchment glue	Sheep	Kremer Pigmente, Germany #63035
8	Sanzenbon Asuka	Cattle	Sankichi, Japan #06-00003
9	Toku Sanzenbon Nikawa	Fish	Sankichi, Japan #06-00042
10	Sturgeon air bladder 7 g*	Sturgeon	PAReT, Japan
11	Sturgeon air bladder 8 g*	Sturgeon	PAReT, Japan
12	Cook gelatin	unspecified	Morinaga, Japan #4902888544019
13	Cooking jerry	unspecified	House Foods, Japan #4902402333198
14	Nippi gelatin	Cattle	Nippi, Japan #KY30
15	Nippi gelatin	Pig	Nippi, Japan #AP-250
16	Nippi gelatin	Fish	Nippi, Japan #FGS-230
17	Kissho gelatin solution	unspecified	Kissho, Japan #4514373600010

* Sturgeon air bladder from different lot

oxidase b (*cytb*) gene [27, 28], rabbit cyclooxygenase-3 (*COX3*) gene [27, 29], and *COI* (fish mitochondrial cytochrome c oxidase subunit I) gene [30]. The PCR enzymes employed in this study were either PrimeSTAR® HS DNA Polymerase (#R10A, Takara Bio, Japan) or MightyAmp™ DNA Polymerase Ver.3 (#R076A, Takara Bio, Japan). The PCR program for animal mitochondrial COI was: 94 °C for 15 s, 46—54 °C for 15 s, and 72 °C for 1.0 min (40 cycles), and those for other species (cattle, sheep, rabbit and fish) were: 94 °C for 15 s, 48—56 °C for 15 s, and 72 °C for 45 s (40 cycles) respectively, followed by agarose electrophoresis visualised by EtBr staining and photocaptured by Lumino Graph I (Atto Co, Japan). The amplified animal COI gene fragment was optionally cloned by Zero Blunt™ TOPO™ PCR Cloning Kit (ThermoFisher Scientific, USA), followed by Sanger sequencing of the amplified DNA region by M13 universal primers (Eurofin Genomics, Luxembourg). The obtained sequences were then subjected to NCBI BLAST search in order for the identifications of the animal species.

Animal species identification of *Nikawa* sourced from *Ukiyo-e*

Ukiyo-e samples (Fig. 3a–d) were subjected to the molecular analysis. *Ukiyo-e* pieces with 4 cm × 4 cm (~ 0.07 g) were prepared respectively by sterile scissors, and immersed in 1 mL TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA) at 70 °C, overnight. The supernatant was carefully transferred to a new tube, and fluorescent dsDNA quantification (Qubit 4) was carried out. Crude DNA extraction was then performed through phenol/chloroform extraction followed by ethanol precipitation. The dried precipitate was dissolved by 1 mL distilled water and subjected to DNA purification using PME Gelatin DNA Kit as described above. The final DNA yields were measured by Qubit 4, respectively.

We attempted PCR analysis with Quick Taq™ HS DyeMix (#DTM-101 Toyobo, Japan) and 1 ng of the purified DNA as a template (94 °C for 15 s, 50 °C for 15 s, and 72 °C for 45 s (55 cycles). The amplification was checked by agarose electrophoresis as described above. For this identification, we used additional primer sets targeting pig *COII* (cytochrome c oxidase subunit II) gene [27, 28], horse *ND5* (NADH dehydrogenase subunit 5) gene [29], deer *cytb* gene [29], goat *Cox3* gene [29] and chicken mitochondrial gene (region extending position 9069 to 9334 including *ATP6* (ATP synthase F_0 subunit 6) gene) [27, 28]. To confirm the reproducibility, the PCR experiments were carried out at least in duplicate.

Results & discussion

Fluorescent Quantification of Intact Nucleic Acids from Gelatins

Aliquots of gelatin solutions sourced from the commercially available gelatins (Table.1) were quantified following the homogenisation. This revealed for the first time that *wanikawa* (gelatin #1–11) except #9 contains more dsDNA than *yonikawa* (gelatin #12–16) (Fig. 1a), approximately 10 times as large amount as those from *yonikawa*. The reason for this great difference might be that the production of *yonikawa* comprises intense purification steps including ion exchange [1–3, 31] (Nippi Inc., personal communication), which might eliminate unbound nucleic acids away from the collagen fibrils, resulting in less nucleic acid contents. Nucleic acids contained in gelatins should be regarded as "impurities" or "contaminant", which would in turn contribute not only to the characteristic physical and chemical features of *wanikawa* [1, 2], but is also accidentally beneficial for conducting molecular biology-based studies like species identification. Regarding the lower nucleic acid content in gelatin #9, we reason that DNAs in gelatin #9, originated from fish scale (devil fish) (Sankichi Co., Ltd., personal communication), might be seceded into the environment during the scale development. Another possibility might be the difference in the collagen solubilisation procedures [3] (Nippi Inc., personal communication). Even amongst *yonikawa* samples (gelatin #13–16), gelatin of fish origin exhibited lower nucleic acid contents (Fig. 1a, b). Fish gelatins are categorised into Type A gelatin, and are generally fabricated through acid method for solubilisation of collagen fibrils [3] (Nippi Inc., personal communication), which might cause irreversible damage on nucleic acids. It would be also analytically intriguing to note that both ssDNA and miRNA are more abundant compared with dsDNA, which will motivate future establishments of molecular biology-based method for identifying species upon ssDNA and miRNA (Fig. 1b). As for gelatin #17 (unknown whether it is *wanikawa* or *yonikawa*), based on the quantified nucleic acids contents (dsDNA, ssDNA, miRNA), we suppose that this gelatin is more likely to be regarded as *wanikawa*.

Species identification through DNA barcoding technique

We then attempted the species identifications through DNA barcoding technique using DNAs extracted from *wanikawa* samples. Gelatins #3 (rabbit), #6 (cattle), #7 (sheep) and #9 (fish) were selected for this step. First, we employed PrimeSTAR® HS DNA Polymerase (Takara Bio, Japan) as PCR enzyme. Specific amplification was observed only in gelatin #6 (Fig. 2a), consistent with the manufacturer's statement about the source of this product. In pursuit of the reason for which only gelatin

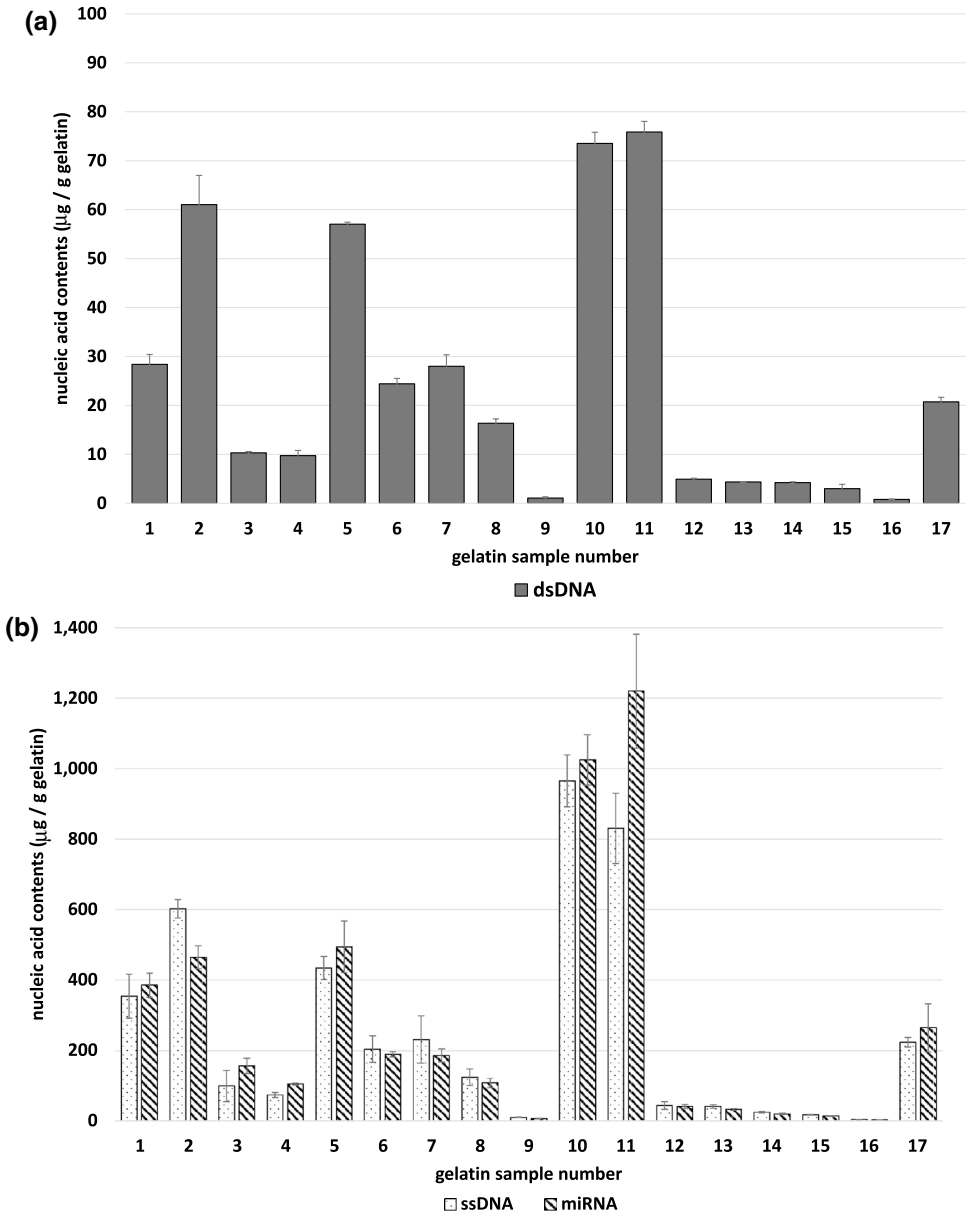

Fig. 1 **a** dsDNA, and **b** ssDNA, miRNA concentration of each gelatin determined through fluorescent assay (n = 3). **a** *Wanikawa* (gelatin #1 ~ 11) in general possess more dsDNAs than *yonikawa* (gelatin #12 to #16). Judging from the dsDNA content, gelatin #14 is more likely to be classified as *wanikawa*. **b** As with the case in dsDNA **(a)**, most of *wanikawa* (gelatin #1 ~ 11) also possess more ssDNAs and miRNAs than *yonikawa* (gelatin #12–#16)

#6 was successful, we suspected the contamination of PCR inhibitory components such as humic acids in the extracted DNAs, even though the extracted DNAs were purified through the commercially available purification kit. Since humins or humic acids are known to be generally included in gelatins [29], we next attempted PCR using MightyAmp™ DNA Polymerase Ver.3, which is notable for its suppression of PCR-inhibitors [32]. As for gelatin #6 (cattle), specific amplification was observed again in the cattle primer set (Fig. 2b), confirming that this species identification system with this PCR enzyme was successful.

Figure 2c shows PCR amplification of gelatin #7 (sheep), in which specific amplification was observed not only in sheep but also in cattle. The reason for the detection of other species' trace or the mixture thereof might be due to the contamination during gelatin manufacturing processes in which gelatins from different species are

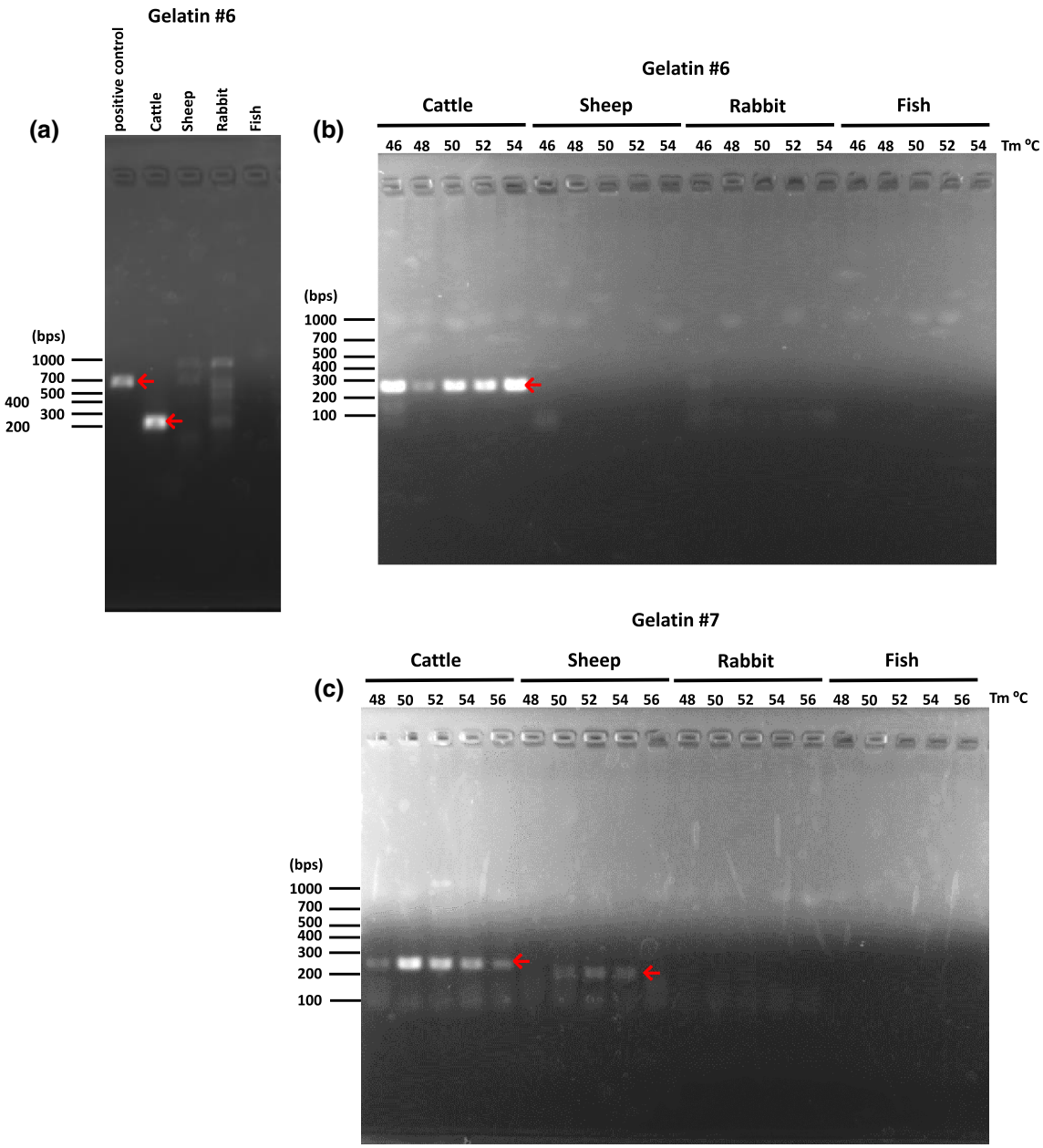

Fig. 2 Species identification through DNA barcoding: **a** gelatin #6 (cattle) with PrimeSTAR; **b** gelatin #6 with MightyAmp DNA Polymerase, gradient PCR; **c** gelatin #7 (sheep) with MightyAmp DNA Polymerase, gradient PCR; **d** gelatin #3 (rabbit) with MightyAmp DNA Polymerase, gradient PCR; **e** gelatin #9 (fish) with MightyAmp DNA Polymerase, gradient PCR. In **a**, animal COI was originally set to be positive control. Upon employing MightyAmp DNA Polymerase, specific amplification was observed (**c, d**), possibly suggesting that *wanikawa* contains inhibitory components for PCR, such as humic acids. From the agarose electrophoresis, it is clear that gelatin #6 is made from cattle (**a, b**), gelatin #7 from cattle and sheep **(c)**, whereas gelatin # 3 from rabbit **(b)**. In contrast, no specific amplification was seen in gelatin #9 **(e)**

boiled up in the same cauldron (Tsumaya Nikawa Laboratory, personal communication). Provided that the cauldron is thoroughly washed up when starting up a new manufacture of another gelatin from different species, residual gelatin combined with the DNAs would have been still stuck to the cauldron, possibly resulting in our detection of both species. Another possibility with regard to partial homologies of sequences between cattle and sheep [33] and/or the contamination of domestic animals proximal to our modern lives [34], which might lead to cross-reaction beyond the target species. However, we suppose that the primer pairs from cattle and sheep

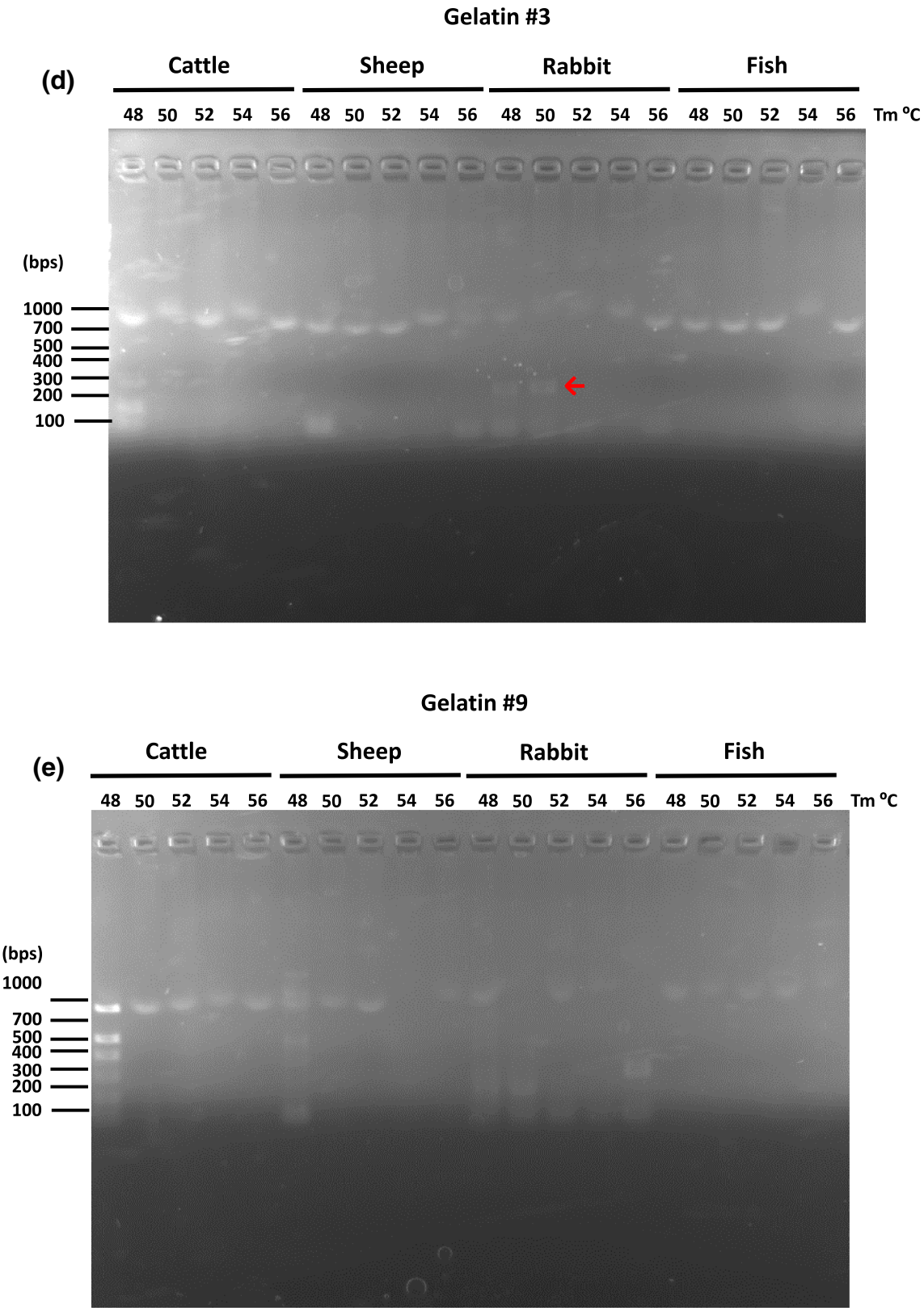

Fig. 2 continued

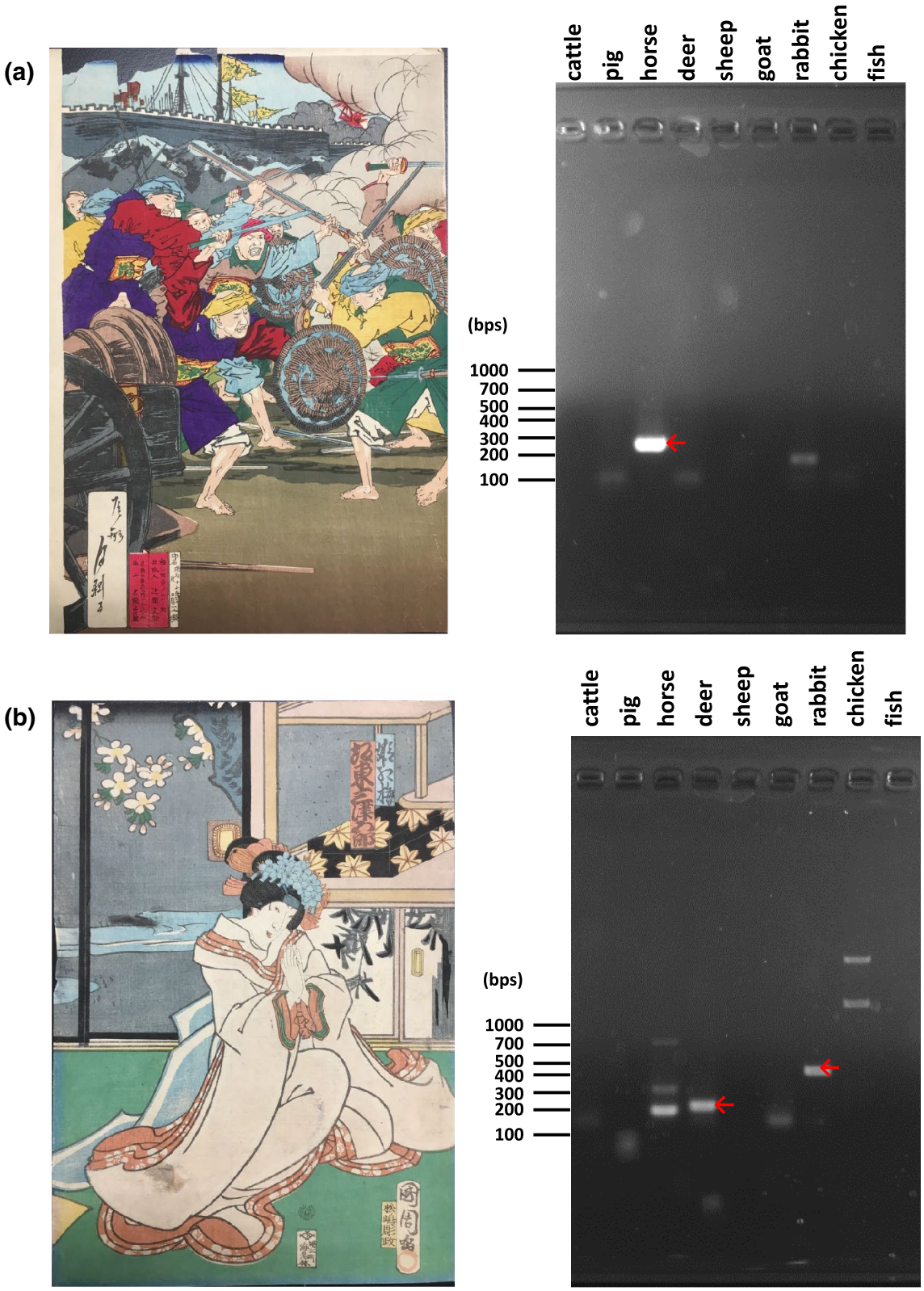

Fig. 3 **a**–**d** *Ukiyo-e* samples analysed in this study. These *Ukiyo-e* were manufactured in **a** 1883 and **b**–**d** in 1864. DNA barcoding study on these *Ukiyo-e* unveiled that the gelatins were sourced from **a** horse, **b** a mixture of deer and rabbit, **c** rabbit, and **d** horse, respectively. The bands that appeared on horse lanes in **b** and **c** were judged to be non-specific products because of the differences from the anticipated band size

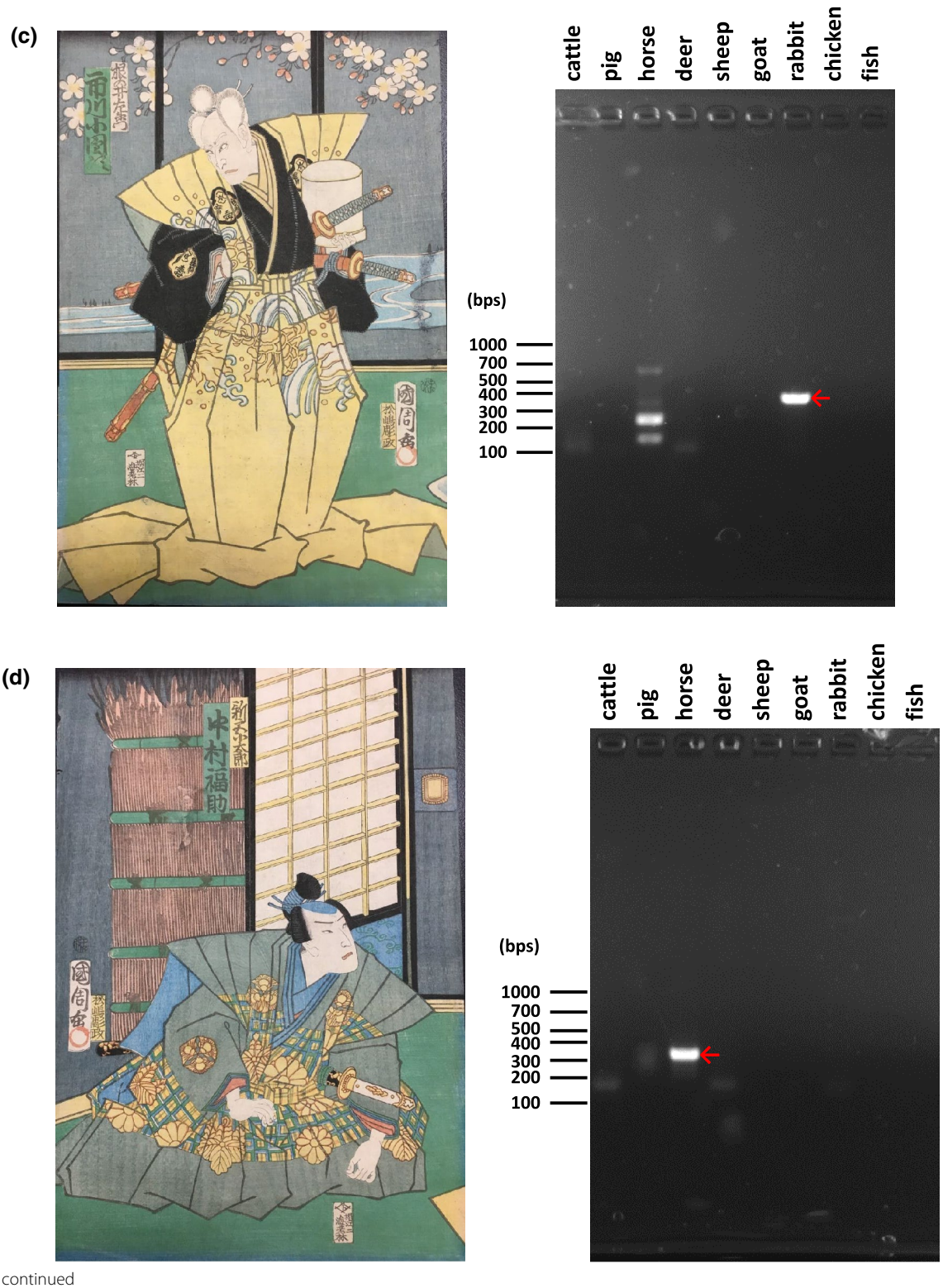

Fig. 3 continued

were unlikely to exhibit a cross-reaction beyond species because each primer pair (Cattle Fw/Rv, Sheep Fw/Rv; Additional file 1: Table S1) is targeted on the different genes respectively with low identities to the other.

Figure 2d is PCR result from gelatin #3 (rabbit), showing that a faint PCR amplification product was observed only in rabbit primer set. Even with the use of the inhibitor-resident enzyme, the PCR trial on devil fish-derived gelatin #9 was unsuccessful (Fig. 2e).

We then attempted species identification for gelatin #9 through the animal COI fragment amplified by the PCR inhibitor-resident PCR enzyme, in which we observed a specific amplification (Additional file 2: Figure S1). The amplified fragments were subcloned, followed by Sanger sequencing. Resulting DNA sequences were subjected to NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This sequence analysis showed that amplified COIs were either from *Homo sapiens* (5 out of 6 trials; data not shown on the ground of ethical care for anonymous individuals) or *Bos taurus* (1 out of 6 trials) with 100% identity respectively.

In addition, we also tested COI gene amplification from gelatin #7 (sheep) likewise as denoted above, revealing that all fragments are from *Homo sapiens* (4 out of 4 trials; data not shown). Whilst the contamination of cattle DNA in gelatin #9 would be possibly attributed to the same reason as discussed above, regarding with the contamination of human DNA, however, we speculate that gelatins are prone to be contacted directly by manufacturers' bare hands during gelatin manufacturing processes, including animal dismantling, tanning, moulding and pouching [1, 2].

These results for COI amplification could lead to the conclusion that use of animal COI gene as a "positive control" would lack the reliability of the molecular biological experiments for species identification, even though comprehensive approaches like amplicon sequencing targeting COI gene were undertaken, at least in the case of gelatin. To overcome this situation, the use of blocking primers capable of preventing PCR amplification from particular species [35] might help. This sort of combination of identification technologies might contribute to de novo extraction of genetic information of archaeological interest.

Practical exemplification of the DNA barcoding technique

We further performed animal species identification of gelatin derived from Japanese classical art called *Ukiyo-e*. For manufacturing classical *Ukiyo-e* or Japanese painting (*nihonga*), gelatin coating using "*dousa*", a mixture of gelatin and alum, has been traditionally applied over the drawing paper ahead of multi-colour woodblock printing procedures for preventing colour bleeding. Gelatin itself has been also utilised for fixing various pigments onto the paper, which motivated us to examine the versatility of the identification method described herein with employing *Ukiyo-e* as the material of interest. Four pieces of *Ukiyo-e* (Fig. 3a–d) were prepared for this purpose. From the printed stamps, these *Ukiyo-e* are proven to have been manufactured in the 16th year of the Meiji era (1883 AD) (Fig. 3a) and the first year of the Genji era (1864 AD) (Fig. 3b–d).

Fluorescent quantification of dsDNA was performed before and after the DNA purification procedures (*i.e.* after TE buffer extraction and after the kit purification, respectively) as described in Materials and Methods, confirming rigid DNA extraction enough for DNA barcoding analysis (Table 2). The differences in dsDNA contents between before and after the purification procedures would account for the dsDNA length distribution summarised in Oshikane et al. [24]. Briefly, whilst dsDNA with at least 4 bps can be detected via the fluorescent quantification, silica resin used for the purification can capture dsDNA with≥100 bps, through which dsDNA with less than ~ 100 bps would have been thus discarded.

Next, Animal species identification was attempted with MightyAmp™ DNA Polymerase Ver.3 as described above, which resulted in no specific amplification. Amongst the differences between the modern and historical *wanikawa*, we here focused on the possibility of postmortem degradation, which often accompanies intrinsic base alteration including cytosine (dC) to uracil (dU) [24, 36, 37]. dU lesion mainly occurs at the ends of DNA strands [38, 39], which would hamper PCR amplification [40, 41] because family B polymerases of archaeal origin are known to harbour uracil binding pocket for DNA repairing [42, 43].

We, therefore, employed the most classical PCR enzyme, Taq polymerase (Quick Taq™ HS DyeMix) categorised into family A polymerase of thermophilic eubacteria origin, leading to successful identifications (Fig. 3a–d) as summarised in Table 2, possibly implying that all of *Ukiyo-e* contain postmortem lesions including dC to dU change. In addition, it is of note that initial DNA extract from *Ukiyo-e* in TE buffer would have

Table 2 Ukiyo-e samples analysed in this study

Ukiyo-e sample	Year of manufacture	dsDNA contents (ng per 4 × 4 cm piece)		Identified animal species
		Before purification	After purification	
a	1864	2560	31	Horse
b	1883	12520	96	Deer, Rabbit
c	1883	15560	118	Rabbit
d	1883	8380	79	Horse

contained PCR inhibitory substances including humic and fulvic acids which will hinder the following DNA analysis. We employed a classical crude extraction procedure comprising phenol/chloroform extraction followed by ethanol precipitation ahead of the kit purification, which is known to confer the removal of the hampering substances to some extent [44], possibly leading to successful amplifications by Taq polymerase.

Conclusion

In this study, nucleic acids encapsulated in *wanikawa* are more abundant than those in *yonikawa* exemplified as shown quantitatively through fluorescent assays. This demonstrates that *wanikawa* according to the classical manufacture (note that the *wanikawa* samples employed in this study could be fabricated recently) are likely to be more suitable for molecular biological studies such as sequence analysis, hopefully providing the future opportunity for molecular analysis on gelatins utilised in cultural artefacts and archaeological samples. Also, we described partial success in animal species identification with DNAs extracted from *wanikawa*. Concerning the PCR for animal species identification, the employment of MightyAmp DNA Polymerase instead of PrimeSTAR led to successful identifications, possibly suggesting that PCR inhibitors such as humic acids are present even after the purification of DNAs from *wanikawa* through the DNA extraction kit. To overcome PCR inhibitors, whilst bovine serum albumin (BSA) has been shown [45–47], we herein reported a novel method for archaeological research by employing inhibitor-resident polymerase. Therefore, in order to conduct molecular analysis on classical gelatins (*wanikawa*), complete removal of PCR inhibitory components via aluminium-based approach [48, 49] or use of PCR enzymes insensitive to PCR impediments [32] as exemplified herein will likely be required.

We also described that DNA barcoding is effective for animal species identification with the DNAs extracted from *wanikawa*. Whilst gelatin #3 (rabbit) and #6 (cattle) were proven to be from rabbit and cattle respectively from the agarose electrophoresis results, gelatin #7 (sheep) intriguingly appeared to be the mixture of cattle and sheep gelatins, at least at DNA level, possibly due to the contamination of DNAs from other origins during the manufacturing processes of gelatins. Since the DNA barcoding primer sets target different genes from each other (see: Materials and Methods), we were not able to judge which species is dominant (*i.e.* origin species of the gelatin) from the quantitative comparison of the amplification yields. Because archaeological specimens in general could exhibit extensive contamination, DNA barcoding methods realising not only qualitative analysis (*i.e.* species identifications) but also quantitative analysis (*i.e.* analysis on the dominant species) would be ideally necessary.

In addition, it would be of archaeological interest to note that the DNA extracted from *wanikawa* appears to contain a considerable amount of human DNA as contaminants. If so, the dsDNA illustrated in Fig. 1a (as well as ssDNA and miRNA in Fig. 1b) might include human-originated nucleic acids. However, since the amplification of the human COI gene was also observed even in the PCR targeting the animal COI gene with *yonikawa*-derived DNAs as a template (data not shown), this sort of contamination could be ubiquitous when it comes to dealing with DNAs extracted from gelatins. This assumption in turn further implies that genetic information about the people who directly dealt with the gelatins, such as butchers, gelatin manufacturers, art painters and craft makers, could be feasibly extracted. As for the physical properties of general gelatins, the glass transition temperature is about 40–45 °C, and the molecular arrangement of gelatins as triplet helices is contribute to tight fibrils [50]. It is therefore conceivable that the DNAs not only originated from the animal species but also from the people could be stably encapsulated in the gelatins at least under standard ambient condition. The captured DNAs would have stayed for a long time with physically preventing endogenous nucleases and damaging chemicals to have retained until today at least enough for DNA barcoding analysis. Given that gelatin generally contains DNAs derived from other species than those of interest, we believe that the contaminated DNAs could perhaps provide valuable clues to elucidate the archaeologically intriguing matters including the ethnic group who had engaged in either the production or use of the gelatins.

Finally, we showed the versatility of DNA barcoding approach by exemplifying species identification of gelatins utilised for manufacturing *Ukiyo-e*. In this case, we employed Taq polymerase for identification. However, as Taq polymerase is generally known to be a lower fidelity enzyme, for conducting further sequential analysis like Sanger or NGS-based sequencing, it would be desirable to choose family A enzyme with higher fidelity. Whereas DNAs from domestic animals especially like cattle, pigs and chicken proximal to our daily lives are prone to be contaminated in the archaeological samples [34], we think that the amplified DNA fragments of horse, rabbit and deer were unlikely from this sort of contamination. We cannot deny, however, the possibility that gelatins from *Ukiyo-e* might also contain fish-derived DNA because the molecular identification of fish was unsuccessful according to this study. From an archaeological point of view, it would be intriguing to pursue the differences in the source of animal species depending upon *Ukiyo-e*. In particular, since three *Ukiyo-e* (b) to

(d) are from the same publisher, there might be technical rationale(s) for the proper use of gelatin.

Recently, proteomics approaches utilising mass spectrometry have been growingly advanced in archaeological science for the identification of animal species [51–55]. Compared with the proteomics approach, this DNA barcoding approach has advantages in realising: (i) multiple species identifications even though the samples of interest were from the mixture of species; (ii) analysis with minute (pico to nano gram-order) DNA samples through PCR amplification; and (iii) further sequential analysis including Sanger and NGS-based sequencing. As for (ii), the 16 cm^2 Ukiyo-e sample yielded ~ 100 ng dsDNA with > 100 bps according to this study. Dilution experiment of dsDNA template from *Ukiyo-e* (a) revealed that amplified products were visible up to at least 2^9 (= 512) dilution under routine EtBr staining (Additional file 3: Figure S2), suggesting that pico gram-order of dsDNA template (*i.e.* 10^{-1} mm^2 order of *Ukiyo-e* sample) would be sufficient for the identification. About (iii), the NGS-based approach has been applied to archaeological science [56], we expect that the DNA barcoding approach combined with comprehensive sequencing technology will shed light on not only the phyletic line of the animals but also the geographic information about the production of the gelatins and the artworks. We think that careful selection of methodology, either the proteomics approach or the DNA approach, should be necessary depending upon the characteristics of the samples (e.g. their rarity, homogeneity and preservation condition).

Above all, we described the archaeologically interesting features of classically manufactured gelatins in view of molecular biological analysis, expecting to pave the way for further establishing molecular biology-based archaeological approaches.

Abbreviations

PCR: Polymerase chain reaction; DNA: Deoxyribonucleic acid; dsDNA: Double-stranded DNA; ssDNA: Single-stranded DNA; miRNA: MircoRNA; ELISA: Enzyme-linked immunosorbent assay; Tris: Tris(hydroxymethyl)aminomethane; EDTA: Ethylenediaminetetraacetic acid.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40494-022-00798-z.

Additional file 1. Primers embodied in this study for the animal identifications.

Additional file 2: Figure S1. PCR trial of animal COI gene with DNA extracted from fish-derived gelatin #9, confirming rigid amplification at Tm = 52 °C.

Additional file 3: Figure S2. Dilution experiment of DNA from *Ukiyo-e* (a). EtBr-stained bands were visible up to 2^9 dilutions.

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Author contributions

AFHO planned the research, HK, MHHO. Did experiments, and HK, YK, KN, TI, TKU, MU, KY, AF, HO drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the starting materials (*i.e. nikawa* samples) exemplified herein can be commercially obtained as illustrated in Table.1. All data analysed in this study are included in the article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consented for publication.

Competing interests

The authors declare no competing interests.

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