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The Effects of Chemical and Heat Maceration Techniques on the Recovery of Nuclear and Mitochondrial DNA from Bone*

ABSTRACT: Forensic anthropologists use a number of maceration techniques to facilitate skeletal analysis of personal identity and trauma, but they may unwittingly eliminate valuable DNA evidence in the process. This study evaluated the effect of 10 maceration methods on gross bone structure and the preservation of DNA in ribs of 12 pigs (*Sus scrofa*). A scoring system was applied to evaluate the ease of maceration and resulting bone quality while DNA purity was quantified by optical densitometry analysis, followed by polymerase chain reaction (PCR) amplification of three mitochondrial and three nuclear loci. The results demonstrated that while mitochondrial DNA could be amplified for all experiments, cleaning treatments using bleach, hydrogen peroxide, ethylenediaminetetraacetic acid/papain, room temperature water and detergent/sodium carbonate followed by degreasing had low DNA concentrations and failed to generate nuclear PCR products. In general, treatments performed at high temperatures (90°C or above) for short durations performed best. This study shows that traditionally "conservative" maceration techniques are not necessarily the best methods to yield DNA from skeletal tissue.

KEYWORDS: forensic science, forensic anthropology, DNA, skeletal preparation, optical densitometry, polymerase chain reaction, maceration

The ability to effectively remove soft tissue from the skeleton without compromising surface morphology or overall bone integrity is paramount to a thorough and complete analysis by a human skeletal biologist. Anthropological interest in maceration techniques began in the 19th century with the collection of skulls from cemeteries for the purposes of craniometric studies. Over the following century, maceration methods evolved as museum curators (1–6), comparative anatomists (7–10), zooarchaeologists (11–16), and human skeletal biologists (17–20) published their methodologies. Perhaps the most ambitious endeavors were the maceration of thousands of individuals to assemble the Hamann-Todd, Terry, and Huntington anatomical collections in the early 20th century. Hunt and Albanese (21) report that Dr. Robert Terry mechanically stripped soft tissue from the bone then placed the bones in hot water for 72 h, followed by drying and treatment with benzene vapors to remove some but not all of the fats from the bone. Terry warned that the complete loss of fats would cause bone to become brittle and unstable. This triad of flesh removal, drying and degreasing is the basic formula of maceration used today.

Maceration is an invaluable procedure in a forensic context, although not all maceration techniques are applicable to human remains in medico-legal cases. Anthropological assessment of a technique's success often revolves around the length and ease of the maceration process, the resulting bone quality and color, and the relative odor. Any method that alters the chemical properties and morphological appearance of bone should be avoided. However, the frequency of submission of human bone samples for nuclear and mitochondrial DNA (mtDNA) testing is steadily increasing in forensic casework, and anthropologists must re-evaluate their maceration methods and criteria for success. Specifically, maceration methods that compromise nuclear and mtDNA must be abolished or crucial evidence may be lost. A previous study provided some insight into heat techniques and DNA degradation of two nuclear loci in two human rib samples (22). The current study is more comprehensive and examined the anthropological and molecular impact of 10 chemical, heat and enzymatic treatments on both nuclear and mtDNA preservation in bone.

Modern Maceration Techniques

There are a large number of maceration recipes, although they can be grouped into six categories: cold water bacterial maceration, warm water bacterial maceration, maceration via cooking, chemical maceration, enzymatic maceration and invertebrate maceration (4,5). Cold water (or room temperature) maceration is traditionally considered the safest method for bone as no heat or chemicals are applied that may disrupt bone integrity, but it is also the most time consuming as it may take weeks for the microbes to reduce the soft tissue (5,14,19,23). Cold water maceration is also notoriously malodorous. Warm water maintained above 37°C is more conducive to bacterial-driven decomposition and takes only hours or days (5). "Cooking" is a somewhat general term for any method that requires additional heat, such as boiling, incubating or microwaving. Bacterial action is no longer a factor, but the high temperatures promote rapid breakdown of the soft tissues.

There are a number of chemical techniques that have been published. Alkaline solutions are preferred as they break down collagen and other proteins. Based on extensive experience with museum collections, Hangay and Dingley (5) reported a solution of warm water and 5% potassium hydroxide that macerates and degreases bones (remove remaining blood and fat) simultaneously,

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although the authors emphasized that the bones must be thoroughly washed with water after treatment to prevent long-term damage. Household bleach (sodium hypochlorite) is also a perennial favorite of museum curators (24), forensic pathologists (25), and anthropologists (18). Hydrogen peroxide removes lipids and is considered less caustic to bone than bleach (26). Hangay and Dingley (5) advocated the use of ammonium and sodium hypochlorite for adult mammalian skulls. Prolonged exposure to any chemical, however, can result in brittle, fragile bones and loss of cortical detail (13). Because some recommended chemicals are flammable or known carcinogens, full personal protective equipment and use of a fume hood are required at all times (27).

Enzymatic preparation often includes laundry detergent or pure papain, pepsin or trypsin in warm or boiling water with or without chemical additives. For instance, Hill (2) suggested adding papain and ethylenediaminetetraacetic acid (EDTA), a chelator, to warm water (45°C), followed by sodium hydroxide or potassium hydroxide to digest ligaments, if necessary. Mooney et al. (10), Nawrocki (23) and Schmid (16) used a solution of laundry detergent and water heated to 75–80°C. In a recent article, Fenton et al. (17) added laundry detergent and powdered sodium carbonate to hot water to remove soft tissue, followed by soaking in a dilute ammonia solution over low heat, which degreases the bones. In addition to ammonia, chemicals such as benzene, gasoline, acetone, hydrogen peroxide and carbon tetrachloride are also useful for degreasing bones following tissue removal (6,16,23–25).

Finally, dermestid beetles and other invertebrate species are useful for reducing tough ligaments and dried tissues while leaving bone intact (6,14,28–30). This method is attractive because there is no risk of chemical damage. However, Anderson (24) pointed out that the beetles are only interested in dried tissue and can take days to weeks to completely consume the soft tissue. In addition, maintaining a colony is difficult, time consuming and expensive. Further, dermestids can live within bone for weeks, requiring extreme temperature treatment, alcohol baths and/or several weeks of quarantine to eliminate all insects from the specimen. Sommer and Anderson (28) suggested dermestid beetles are best used for small specimens that tend to be destroyed by other maceration methods. Allen and Neill (11) claimed mealworms (tenebrionid beetle larvae) provide a good alternative to dermestid beetles as they are easily acquired from pet stores, cost little, require less attention, and do not present the same risk of infestation. However, more recent references on the use of mealworms as a maceration technique have not been found.

Clearly no single method is a silver bullet for all situations. Bleach cleans and whitens bones, which may be appropriate for museum display, yet can also result in cortical exfoliation that would hinder examination of forensically important surface defects. Similarly, hydrogen peroxide and enzymatic laundry detergent can also alter the cortical surface and leave bones chalky (24,25). Boiling can cause teeth and bones to scorch, split and crack, especially if boiling is too rapid or extensive (23,24). Careful treatment, no matter what the method, can usually forestall such complications; forensic anthropologists continue to use a wide variety of techniques. The larger issue examined here is whether maceration methods employed by anthropologists are inadvertently compromising DNA integrity and thereby destroying potential physical evidence.

Materials and Methods

Fresh ribs from 12 pigs (*Sus scrofa*) were obtained from a local butcher. Sets of three adjacent ribs connected by soft tissue and with cartilage intact were weighed and then placed in one of 10 experimental conditions plus a control. Except for detergent followed by degreasing, each experiment was repeated a minimum of three times using ribs from different individuals. Chafing dishes and hotplates were used for heating. Solutions were changed at least once daily except for the experiments carried out at room temperature, which were changed weekly, since frequent water replacement reduces bacteria. With exception of the microwave method, all maceration took place under a Fisher Hamilton Safe Aire® 54L series fume hood (Two Rivers, WI).

Maceration Methods

Ten maceration techniques were elicited from the published and unpublished literature as well as from personal communications with active forensic anthropologists. Loose soft tissue was removed by gentle pushing whenever the solution was changed, but mechanical removal was kept to a minimum for each experiment. Except for one treatment, no additional degreasing chemicals were applied following maceration. Once cleaned, bones from each experiment were washed thoroughly with water before drying.

- *Mechanical Removal of Flesh*: This was used as a control whereby the ribs were manually defleshed using scalpels and forceps to remove meat and strip the periosteum. Care was taken to avoid scraping, chafing, or cutting bone.
- *Room Temperature Bath*: A minimum of 10.5 L of water was kept at room temperature (*c*. 22°C).
- *Hot Water Bath*: Approximately 10.5 L of water was maintained at or just below 90°C.
- *Boiling*: Samples were placed in *c*. 10.5 L of water and brought to a boil that was maintained around 100°C.
- *Microwave*: Specimens were placed in a microwave-safe dish and loosely covered with a lid or plastic wrap to prevent dehydration. Samples were heated in a 1300 W, 2450 MHz microwave oven (Sears, Hoffman Estates, IL) on high power for 1 min intervals until all flesh easily slipped from the bones.
- *Bleach (Sodium Hypochlorite) Bath*: A 10% bleach (Clorox, Oakland, CA) solution (1.05 L liquid bleach and 9.45 L water) was kept at room temperature.
- *Hydrogen Peroxide (H_2O_2)*: 1.0 L 3% hydrogen peroxide (Cumberland Swan, Smyrna, TN) in 9.5 L water was kept at room temperature.
- *EDTA and Papain*: $2\frac{3}{4}$ teaspoon (11.25 g) EDTA (Fisher Scientific, Fair Lawn, NJ) and $2\frac{3}{4}$ teaspoon (13.6 g) papain (Sigma, St. Louis, MO) per 10.5 L water was heated but maintained below 45°C.
- *Meat Tenderizer and Palmolive®*: Six teaspoons (39.4 g) Adolph's® (Lipton, Englewood Cliffs, NJ) nonseasoned meat tenderizer and 6 teaspoons (29.6 mL) Palmolive® (Colgate/Palmolive, New York, NY) per 10.5 L water was maintained at or below 90°C.
- *Detergent/Sodium Carbonate*: A solution of 7 tablespoons (100 cc) powdered Biz® (Redox Brands, Inc., West Chester, OH) and 7 tablespoons (100 cc) powdered sodium carbonate (Arm and Hammer Super Washing Soda™, Church and Dwight Co., Inc., Princeton, NJ) per 10.5 L water was maintained at or below 90°C.
- *Detergent/Sodium Carbonate Followed by Degreaser*: The detergent/sodium carbonate procedure was followed as above. Bones were rinsed thoroughly and then placed in a solution of 300 mL liquid sudsy ammonia and 4 L water.

A scoring system was established to quantify odor, soft-tissue texture, ease of soft-tissue removal, and bone quality (Table 1). These observations and water temperature were recorded three to four times a day, the latter with an Enviro-Safe® laboratory thermometer (H-B Instrument Company, Collegeville, PA). Observations of the microwave method took place every 60 sec. A final score for each variable was calculated as the average score across all observations. Bone quality was assessed by comparing dried ribs with those mechanically macerated from each individual.

Bone Preparation for DNA Analysis

Following completion of the maceration phase of the experiment, each sample was air dried on quarter-inch mesh screens at room temperature for at least 2 days and then weighed. Extraction of the bone core samples from the ribs took place under a fume hood. Prior to extraction, the cortical surface of each sample was removed using a Sears Craftsman Dremel® tool with a coarse sanding bit. The cancellous bone was then extracted using a combination of both the Dremel® tool and a Makita® drill (Buford, GA). The samples were pulverized using a stone mortar and pestle. Approximately 0.7 g of cancellous bone tissue was collected from each specimen. To avoid cross contamination of the samples, all surfaces and equipment were cleaned with a 10% bleach solution prior to each extraction.

TABLE 1—*Maceration scoring system.*

Odor
1. Strong smell that permeates the entire lab space
2. Moderate smell in the immediate vicinity of the experiment
3. Little to no smell; a slight odor possible around or under the fume hood

Soft-tissue texture
1. Soft tissue is firm and/or quite solid; may feel tougher or more rubbery than when first submerged
2. Soft tissue is as malleable as when originally observed
3. Soft tissue is considerably softer and looser than when the experiment began; very malleable
4. Soft tissue is nearly liquefied and floats on the surface with little or no connection to bone

Ease of flesh removal
1. Adherence to bone is quite strong with little or no flesh removal possible without damaging the bone
2. Adherence to bone is moderately strong although large portions can be easily removed; the core of flesh close to the bone is still adherent
3. Adherence is minimal to bone as flesh falls off as bones are removed from solution or easily removed with fingertips

Bone quality
1. Brittle, fragile, easily broken
2. No cortical erosion but bone is lighter in weight and porous
3. Softer, more pliable than normal bone but no cortical damage
4. Cortex eroding and/or flaking but bone will not easily fracture
5. Strong, normal bone texture and quality

DNA Extraction

Bone decalcification as well as DNA extraction and purification were accomplished by following the FBI protocols for bone (31). Products were then run on 1% agarose gels, stained with SYBR gold® nucleic acid gel stain (Molecular Probes, Eugene, OR) and visualized using the Kodak EDAS gel system (Kodak, Rochester, NY). Total DNA concentration was measured by spectrophotometry (GeneQuant II system, Amersham Pharmacia Biotech, Cambridge, UK) at 260 nm. Purity of DNA was assessed using the ratio of $OD_{260/280}$ with a ratio of 1.7–2.0 representing good purity (32).

PCR Amplification of Mitochondrial and Nuclear DNA

Total DNA from the pig rib bone samples was used as template DNA for PCR-based amplification. Success of the 10 maceration conditions was evaluated in part based on the ability to amplify three nuclear and three mitochondrial PCR products (Table 2). These six loci, ranging in size from 268 to 750 bp, were chosen randomly from the National Center for Biotechnology Information (NCBI) GenBank database (33) in order to assess the overall purity of the DNA extracted from the bone samples from each maceration technique.

PCR amplification was performed on a Perkin-Elmer GeneAmp® PCR Systems 2400 (Norwalk, CT) in a 15 μL reaction volume containing 0.76 μL of template DNA, 0.3 μL of each 5 μM primer and 13.64 μL of Platinum PCR Supermix® (Invitrogen, Carlsbad, CA), which includes reaction buffer, dNTPs, $MgCl_2$, *Taq* DNA polymerase, and anti-*Taq* DNA polymerase. PCR reactions were run with a 3 min denaturation step at 94°C followed by 34 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. Products were separated by electrophoresis on a 2% agarose gel along with a 100 bp ladder (Invitrogen) as a size marker. The gels were then stained with SYBR gold® and photographed under UV light using a Kodak EDAS gel system. Each PCR reaction was repeated at least four times for all samples.

TABLE 2—*Nuclear and mitochondrial sequences for* Sus scrofa.

Primer	Type (bp size)	Origin (GenBank Accession Number)	Forward Primer Sequence	Reverse Primer Sequence
COL	Nuclear (440)	Collagen type V alpha 2 (AY368623)	5′-CAT CTC TAG AAG CTG GGA TGG ACT-3′	5′-GTT GTG TGC ATG GCT TGT CCT T-3′
TREM1	Nuclear (268)	Triggering receptor (AY382476)	5′-GCT GCT TTG GAT GCT CTT CAT CAC-3′	5′-TCT CTC TCA GCT GAC AAG GAC CTA-3′
Osteo	Nuclear (503)	Osteoclast stimulating factor (AF523268)	5′-TTC CAA AGG CAG GAC TGG ACT GAT-3′	5′-ACA CTC CTC TGG CAA AGA CAA GGT-3′
D-loop	Mitochondrial (750)	Mitochondrial D-loop (AF535164)	5′-ATC TCG AGC TTA ACT ACC ATG CCG-3′	5′-GCA AGG CGT TAT AGG GTG TGT AGA-3′
Cyt B	Mitochondrial (420)	Cytochrome b (AY237533)	5′-TCA CAC GAT TCT TCG CCT TCC ACT-3′	5′-TGA TGA ACG GGT GTT CTA CGG GTT-3′
16S	Mitochondrial (497)	16S ribosomal RNA gene (AY243487)	5′-TAA CGA GCC TGG TGA TAG CTG GTT-3′	5′-GGT TTG TGT TTG CCG AGT TCC CTT-3′

TABLE 3—*Anthropological results of experimental maceration.*

Experiment*	Sample Size	Start Weight Range (g) (Average)	End Weight Range (g) (Average)	Average Time	No. of Observations†	Average Odor	Average Texture	Average Ease	Average Bone Quality
Room temperature (22°C)	4	363–522 (441)	14–49 (29)	14–34 d (23.9 d)	90	2.4	2.4	1.4	5
Hot water (90°C)	5	434–716 (548)	13–66 (36)	5.1–27 h (14 h)	23	3.0	2.1	1.8	4.8
Boiling (100°C)	7	402–670 (499)	13–44 (27)	2–5 h (3 h)	22	3.0	2.8	2.7	5
Microwave (high)	5	246–725 (442)	14–38 (25)	5–25 m (14 m)	72	2.3	2.2	1.8	4.6
Bleach (22°C)	4	222–562 (442)	4–50 (31)	12–70 d (33 d)	171	2.4	2.0	1.3	1.25
Hydrogen peroxide (22°C)	4	293–580 (440)	20–54 (30)	23.5–34 d (30.7 d)	88	2.1	2.6	1.5	4.25
EDTA/Papain (45°C)	5	383–690 (561)	25–49 (32)	3.9–28 d (10 d)	146	2.86	2.4	2.1	4.8
Adolph's/Palmolive (90°C)	6	211–740 (447)	5–44 (23)	3–27 h (9.2 h)	15	2.7	2.9	4.0	5
Biz/Na_2CO_3 (90°C)	3	382–592 (480)	18–26 (21)	3–4.2 h (3.7 h)	11	3.0	2.9	1.3	3.7
Biz/Na_2CO_3 degreased (90°C)	2	306–317 (311)	7–10 (8.5)	6.5–7.4 h (7 h)	7	3.0	2.4	1.7	2

*Target temperature given.
†Total number of observations.
d, days; h, hours; m, minutes.

Results

The maceration methods were first evaluated based on the efficiency of the process and gross quality of the bone, followed by an analysis of DNA quality and quantity.

Maceration

The control group consisted of ribs from which the soft tissue was mechanically removed without heat or chemical intervention. The structural quality of the resulting bones was excellent although they were uniformly reddish brown and greasy since blood and fats were still present. The results of the experimental macerations showed that procedures lacking heat (room temperature water, bleach and hydrogen peroxide) or were only heated slightly (EDTA/papain) were substantially slower than hot and boiling water techniques, generally more malodorous, and did not necessarily produce better quality bone (Table 3). Soaking the remains in bleach, hydrogen peroxide solutions, or water at room temperature proved to be the slowest techniques, taking up to 2 months to complete. For bones treated in the hydrogen peroxide solution, the end result, however, was fairly good as the bones were very clean and white, although some exfoliation at the site of muscle attachments was observed. The bleach solution was slightly more efficient but still took a minimum of 12 days, and one experiment lasted 70 days. The bones were white, but not uniformly so, and were very porous at the sternal ends. The cortices were chalky and easily flaked with the slightest impact. Despite the long maceration time, the gross structural quality of bone macerated in room temperature water was good and the bones were uniformly tan with no dark discolorations. Finally, the EDTA/papain solution had a shorter completion time (average of 10 days), although in one case the odor became quite offensive. This solution did not remove cartilage as effectively as other techniques. The resulting bone was brown with dark stains that could obscure observations of surface features, such as cutmarks and antemortem periosteal reactions.

Elevated temperatures expedited soft-tissue removal, as all heat-related methods (hot water, boiling water, microwave, Adolph®/Palmolive®, and detergent/sodium carbonate) were complete within hours. For the same temperature, the detergent/sodium carbonate solution produced more rapid results (average 3.7 h) than meat tenderizer and dish soap (average 9.2 h), but bone quality was scored slightly lower for the detergent/sodium carbonate method because of loss of bone density and slight exfoliation of the cortex. Both procedures produced more rapid results than hot water alone (average 14 h), but were slower than boiling water alone (average 3.0 h), which produced light tan or white bones without discoloration or exfoliation. However, the Adolph's®/Palmolive® solution may offer some advantages over hot water solutions alone in terms of soft tissue breakdown, ease of removal and cleanup. This solution quickly produced a soft meat texture and loosened the periosteum such that it easily slipped from the bone with little manual pressure. The soft tissue did not liquefy but rather came off the bone in a patent mass, which expedited cleanup. While papain and other enzymes in the meat tenderizer were likely denatured when temperatures climbed towards 90°C, the soft tissues still became more pliable earlier than in the EDTA/papain experiment held at lower temperatures.

The microwave technique also resulted in bone with good gross bone structure. Depending on the starting weight of the sample, complete maceration took between 5 and 25 min. The relatively low scores for texture and ease of removal are somewhat misleading as there was little gross soft-tissue texture change until the last minute of each experiment when the meat simply slid off the bone cleanly after it was cooked. Similarly, the odor scores demonstrate that the smell of cooking meat did waft beyond the immediate work area but, in practice, the microwave could be placed under a fume hood. It is important to keep the sample moist while heating by adding water and covering tightly to keep the bones from drying out as the flesh retracts. Drying of the exposed sternal ends resulted in a lower bone quality score for one rib set, although it took only 5 min to process.

DNA Extraction and Amplification

Visualization of total extracted DNA using agarose gel electrophoresis demonstrated that no method of maceration yielded completely intact DNA, as only smeared products were observed (Fig. 1). However, the optical density values (Table 4) suggest that five maceration techniques—treatment with EDTA/papain, bleach, hydrogen peroxide, room temperature water, and detergent/sodium carbonate followed by degreasing—yielded lower

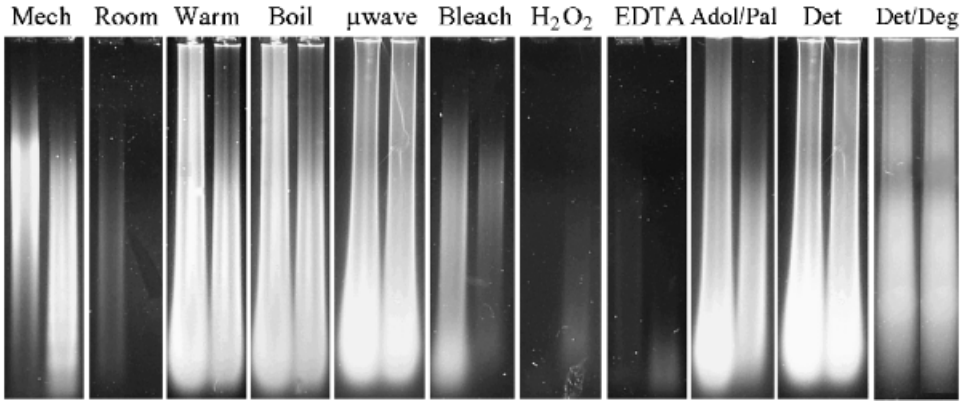

FIG. 1—*Nuclear DNA extracts from pig bones following 10 maceration techniques, including mechanical removal of flesh (Mech), soaking in room temperature water (Room), soaking in 90°C water (Hot), boiling in water (Boil), microwave treatment (μwave), soaking in bleach (Bleach), soaking in hydrogen peroxide (H_2O_2), treatment with ethylene diamine tetraaacetic acid (EDTA) and papain (EDTA), treatment with Adolph's® meat tenderizer and Palmolive® (Adol/Pal), treatment with detergent (Det), and treatment with detergent followed by degreasing (Det/Deg). Following extraction, DNA samples were run on a 1% agarose gel and visualized by staining with SYBR gold®. Representative gel from a minimum of three separate experiments.*

TABLE 4—*Yield and quality of bone DNA isolated following different maceration conditions.*

Maceration Condition	Yield (μg/μL)*	OD_{260}/OD_{280} Ratio*
Mechanical	0.82 ± 0.09	1.846 ± 0.13
Room temperature water	0.00 ± 0.00	NA
Hot water	1.87 ± 0.08	1.786 ± 0.09
Boiling water	1.22 ± 0.28	1.705 ± 0.03
Microwave	0.95 ± 0.50	1.733 ± 0.12
Bleach	0.10 ± 0.00	NA
Hydrogen peroxide	0.00 ± 0.00	NA
EDTA/Papain	0.06 ± 0.05	NA
Adolph's®/Palmolive®	1.17 ± 0.19	1.776 ± 0.08
Detergent/sodium carbonate	1.60 ± 0.14	1.743 ± 0.11
Detergent/sodium carbonate followed by degreasing	0.23 ± 0.08	1.899 ± 0.09

*Results are presented as the mean ± standard deviation from two experiments.

NA, not applicable.

DNA concentrations than did the remaining experimental conditions. The maceration techniques that yielded the highest concentration of DNA were those that involved high temperatures and short durations—hot and boiling water, microwave, Adolph's®/Palmolive®, and detergent/sodium carbonate without degreasing.

PCR amplification results of the three nuclear loci were consistent with the concentration of DNA that had been calculated from the optical densitometry results. Bone treated with bleach, EDTA/papain, or detergent/sodium carbonate and degreasing solutions did not yield nuclear PCR products (Fig. 2). Faint bands were seen for two DNA loci obtained from bone treated with hydrogen peroxide, and one DNA locus (osteo) was attained from bone treated in room temperature water despite its larger size relative to the other loci. PCR amplification was successful for maceration techniques that, with the exception of detergent/sodium carbonate followed by degreasing, took a few minutes (microwave) to, on average, less than a day (Adolph's®/Palmolive®, detergent/sodium carbonate, hot water, and boiling water).

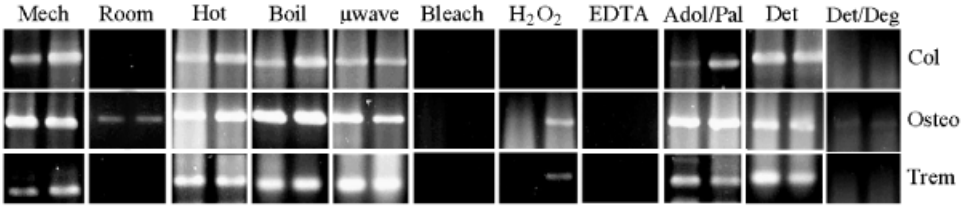

FIG. 2—*Polymerase chain reaction (PCR) amplification of DNA extract of three nuclear loci from pig bones (Table 2). Bone treatments (column headings) are the same as those in Fig. 1. PCR products for the indicated isolates were resolved on a 2% agarose gel and stained with SYBR gold®. Representative gel from a minimum of three separate experiments.*

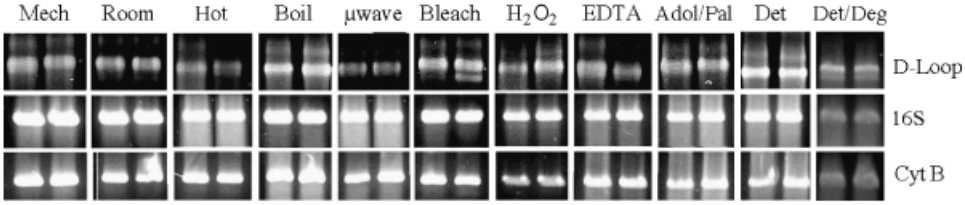

FIG. 3—*Polymerase chain reaction (PCR) amplification of DNA extract of three mitochondrial loci from pig bones (Table 2). Bone treatments (column headings) are the same as those in Fig. 1. PCR products were resolved on a 2% agarose gel stained with SYBR gold®. Representative gel from a minimum of three separate experiments.*

While variability was observed between samples using nuclear primers, no differences were observed between the different maceration techniques when three mitochondrial loci were amplified (Fig. 3). MtDNA amplification was successful even for cases in which no DNA was detected by optical densitometry (Table 4), suggesting that sufficient mtDNA was extracted to generate products following PCR amplification.

Discussion

The ideal maceration techniques will quickly and efficiently remove soft tissue and provide clean, degreased bones with intact mitochondrial and/or nuclear DNA. Fortunately, the maceration techniques that proved most efficient in terms of completion time, ease of processing and rib bone quality (Adolph's®/Palmolive®, detergent/sodium carbonate, hot water, boiling water, and microwave) also yielded the highest amounts of total DNA, as determined by optical densitometry. The most lengthy maceration procedures (room temperature, hydrogen peroxide, bleach, and EDTA/papain) resulted in low recovery of DNA and lack of amplification of nuclear PCR products. These results suggest that lengthy aqueous processing, no matter what the solution, has a deleterious effect on nuclear DNA recovery. While bone treated with detergent/sodium carbonate in hot water did yield PCR results, continued treatment with ammonia as a degreaser appears to have negatively impacted the PCR product, although the sample size is currently too small to determine whether ammonia and/or time is responsible. However, successful PCR amplification of small fragments of nuclear DNA has been reported from human skeletal remains that have been immersed in water for up to 3 years (34–36). Clearly the factors involved in long-term water submersion and DNA recovery require additional study.

A previous study of maceration techniques on the recovery of nuclear DNA from human ribs suggests that heat methods are deleterious to DNA recovery (22). Our results show quite the opposite. Treatment of bone for short durations at high temperatures appears to be not only the most effective way to remove soft tissue, but also maintains rib bone and DNA integrity. However, to better study DNA degradation, increasingly larger mtDNA and nuclear loci should be analyzed until a size is reached at which amplification is no longer possible (37). Such an investigation may also help explain why a relatively large nuclear locus (osteo) was more successfully amplified than smaller nuclear loci in this study. Further, the effects of these maceration techniques on DNA recovery of other bone types (e.g., femora) are not known since only ribs were used in this study. Finally, studies on human bones are also clearly necessary, although such experiments are beyond the scope of the current study.

By utilizing pig ribs and rating the maceration methods only on general bone structure and surface exfoliation that may hinder analyses of trauma and antemortem pathological conditions, the results of this study do not address the impact on more delicate skeletal structures. Suchey and Katz (38) reported that chemical techniques result in loss of fine cortical detail crucial to the examination of human pubic symphyseal morphology for age estimation and advocated slow soaking processes. Therefore, we recommend removal of the pubic bones and other analytically sensitive skeletal regions prior to chemical maceration treatments.

While this study assessed bone and DNA quality within a few days following maceration, there is potential for long-term damage to bone and nucleic acid integrity because of enzymatic and chemical techniques (5,17). For instance, Shelton and Buckley (39) found that a collection of fish bones macerated in a heated enzymatic detergent solution became extremely brittle after several years. The authors attributed this condition to continued enzymatic action on collagen well after treatment had been terminated. Bleach and hydrogen peroxide can also continue to degrade bone after processing (5). Despite a protocol of liberal rinsing of the bones after maceration, the long-term effects on DNA should be noted, especially since many forensic anthropological cases become "cold cases" that may be reopened for DNA testing years later.

Although tangential to the current study, another important ramification of certain maceration methods that is often overlooked may be the effect on bone size and shape. Williams and Smith (40) found that temperatures above 90°C caused loss of bone weight, resulting in changes in some dimensions of nonhuman crania. If decalcification does occur and leads to gross shape changes in human skulls, morphometric analyses, such as the FORDISC programs (41), could be impacted.

Conclusions

The goal of this study was to identify the most effective techniques of maceration that concomitantly permit extraction of the least degraded DNA from bone and their subsequent amplification by PCR. We find that relatively acute periods of high temperature may be the best strategy to achieve both goals. Recommended methods include hot water, boiling water, microwave, detergent/sodium carbonate, and Adolph's®/Palmolive® as they yielded the highest concentration of DNA extract and allowed the amplification of both nuclear and mitochondrial loci. Adolph's®/Palmolive® is an easily attainable and cost-effective alternative to commercial grade EDTA and papain and performed better in this study. Bleach was the worst maceration technique in terms of both bone quality and subsequent DNA purity as too little nuclear DNA was extracted for amplification. While museum curators may desire very white bones for display purposes, the exhibition of human remains is not the focus of forensic anthropology, and we recommend that bleaching techniques be avoided in forensic cases.

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