INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 4, Issue 2, April-June 2013: 127-132 www.ijcs.uaic.ro

CONSERVATION OF MITOCHONDRIAL DNA IN FAST ENZYME-MACERATED SKELETAL MATERIAL

Anne Marie ERIKSEN, Kim Pilkjær SIMONSEN, Arne Redsted RASMUSSEN*

School of Conservation. Esplanaden 34, DK-1263 Copenhagen K, Denmark.

Abstract

The current study investigates the conservation of mitochondria DNA (mtDNA) from skeletal material prepared by enzyme maceration. Ten individuals of Stone Marten (Martes foina) were enzyme-macerated using a mixture of protease and lipase. After using a fast enzyme preparation method the skeletal material was stored for two years in order to see if degradation of DNA had taken place. As warm-water maceration is the traditional maceration technique used for example at The Natural History Museum of Denmark, ten different individuals of Stone Marten were warm-water macerated so a comparison of the two different maceration techniques' amplifiable DNA outcome could be made. Samples for DNA analysis were taken from two different kinds of bone (pelvic and costa) from each individual. The analyses showed that the mtDNA was intact and all PCR products could be indentified to the right species without contamination, demonstrating that both the warm-water maceration and the fast enzyme preparation method had not compromised the DNA.

Keywords: *forensic science; mtDNA; conservation; enzyme maceration; skeletal material; bones*

Introduction

For many years natural history museums have practiced the cleaning of bones for scientific studies, exhibitions and dry storage, but also the fields of forensic science, anthropology and taxidermy have been using different techniques to clean skeletal material [1-7]. Mostly insect consumption and cold- or warm-water maceration have been used as standard maceration techniques [4, 8-12]. Most of these methods require between 2 days and 8 weeks depending on the amount of bacteria present, the size of material being macerated and the temperature used during the maceration [4, 13]. Boiling and subsequent mechanical cleaning of skeletal material is also used, however, such heat treatment may compromise surface morphology [14] and in future research affect the ability to retrieve useful DNA from the material [12, 15-18]. Also detergent maceration has been used for years as a cleaning agent [2, 3, 14, 16, 19-21]. However, information on the exact composition of commercial detergents used is normally not available. It is also worth noting that various kinds of detergents contain tensides, builders (inorganic complexing agents), additives, bleaching agents, and corrosion inhibitors [20, 22-25]. The aggressive mixture in detergents may cause damage to the bones, and softening, decalcification, and transparency of detergent-macerated bones have thus been

* Corresponding author: arr@kadk.dk

observed [3, 24, 25].

Recent results using commercial proteases and lipases to perform enzyme maceration proved to be remarkably fast compared to the traditional methods used [26]. This method allows preparation of skeletal material in an essentially odourless way within a matter of hours, making the method useful not only in natural history museums, but also in forensic science, in private conservation workshops and for educational purposes [26].

The development of DNA extracting methods is progressing fast. With the invention of the polymerase chain reaction (PCR) and second generation sequencing [27], DNA research has progressed from the retrieval of small fragments of mitochondrial DNA to large-scale studies of ancient populations and even complete mitochondrial as well as nuclear genome sequences [28-30]. The PCR technique is an extremely sensitive method that can detect minute amounts of specific DNA molecules and amplify these molecules billions of times in a few hours.

The increasing use of DNA in biology, conservation, forensic casework and in science in general does not allow maceration methods that destroy the use of DNA extraction. Therefore, in the current study we have investigated a traditional skeletal maceration, warm-water maceration, as a large part of the skeletal material found in the storage facilities and in exhibitions today has been macerated this way, together with the enzyme maceration method published by Simonsen et al. [26]. This was done in order to examine whether the traditional maceration method and the one using these enzymes have any impact on extracting useful mtDNA from the treated bones.

Material and Methods

Ten different individuals of Stone Marten (*Martes foina*) were enzyme-macerated using a solution of protease and lipase. Each individual was skinned and the organs were removed before the remains were secured in gauze and macerated for 5 hours at 55°C ± 5°C. The maceration bath consisted of 2.9L of tap water, 50mL of Savinase® 16L Type EX and 50mL of Lipex® 100L, giving a concentration of protease and lipase around 22 and 11µM, respectively. The completion of the maceration was defined as the point in time, with resolution of 15min intervals, where the skull and jaw were separated and tongue and cerebral matter were dissolved [26]. Each specimen was stored in a separate plastic bag at the laboratory at ambient temperature.

Ten other individuals were skinned, the organs were removed, and the remains were macerated in warm water (37°C) for one week. The maceration was brought to an end by boiling the bones for half an hour in a mixture of water and Na_2CO_3. Thereafter the bones were kept in a solution of water and H_2O_2 for 12 hours. The bones were cleaned in fresh water and dried.

As DNA yields can vary greatly between bone types [16], two different kinds of bone material (pelvic and costa bones) from the 20 different individuals were investigated.

Two years after the enzyme maceration and one week after the warm-water maceration DNA was extracted from the skeletal material using DNeasy Blood & Tissue Kit (QIAGEN) following the supplier's manual. Various precautions were taken in reducing the contamination hazard. Two sets of blank control samples were made, one purification blank and one PCR blank. Different rooms for drilling, extraction, and PCR were used and the two different kinds of bone material were processed in two different laboratories (pelvic at the School of

Conservation DNA laboratory and costa at the Natural History Museum of Denmark, Laboratory of Molecular Systematic). Two different sets of carnivore specific primers were selected. One designed to amplify a short fragment length (approximately 200 bp) on the mtDNA control region and another designed to amplify a longer fragment length (approximately 350 bp) on cytochrome *b* [11, 31]. The DNA extraction was first done with the short primer in order to determine whether any DNA was preserved in the bone. The longer primer was used to ascertain that longer fragments of DNA were also preserved.

A standard double-stranded 50µL PCR was carried out using 1µL of the extracted DNA as template. As mentioned above the first primer selected was designed to amplify a fragment of approximately 200bp on the mtDNA control region. The forward primer LRCB1: (5'-TGGTCTTGTAAACCAAAAATGG-3') and the reverse primer MARDH: (5'-CATGCTTATATGCATGGGGC-3') [31]. This primer set was used on the ten samples extracted from the pelvic bones. Thermal cycling was performed on an ESCO swift MinePro, Buck & Holm. The PCR-conditions for the fragment were: One initial cycle of denaturation (94°C for 10 minutes), followed by 35 cycles (94°C for 1 minute, 50°C for 1 minute and 72°C for 1½ minutes) and finally 10 minutes extension step at 72°C [31]). The second set of primers selected was designed to amplify a fragment of approximately 350bp on cytochrome *b*. The forward primer Cb-MM1: (5'- ATGACCAACATTCGTAAAACT-3') and the reverse primer Cb-MMR4: (5'-CCAATGTTCCATGTTTCGGG-3') [11]. The thermal cycling with the second set of primers was performed on a BioRad, PTC 200. The PCR-conditions were the same as for the first primer set, except the annealing temperature was increased to 54°C.

The PCR products were visualised on a 2% agarose gel containing SYBR Safe DNA gel stain SYBR green. The PCR products were purified using QIAquick Purification Kit (QIAGEN) for all the primers. The purified PCR products were used as templates for a 12µL cyclic sequencing reaction using ABI prism® BigDye® Terminator v1.1 Cycle Sequencing kit. For the cyclic sequencing reaction the same primers and PCR conditions were used as for the two initial PCR reactions. The cyclic sequencing PCR was carried out on a gradient Cycler, BIO-RAD, DNAEngive, Peltier Thermal Cycler. The purified sequencing products were run on a 3130xl Genetic Analyser Applied Biosystems HITACHI and the attached computer with Genetic Analysis Program. The sequences were assembled using the computer program CLC Main Workbench 5 and submitted to NCBI nucleotide collection for comparison of the sequences with sequences held at NCBI. The obtained results were used to identify the species of DNA in the different samples.

Results and discussion

No contamination was evident for either the DNA extraction control or the PCR amplification control. Using the two primer sets it was possible to obtain positive amplification products for the entire range of test material. DNA was successfully extracted from all the bones. During the gel electrophoresis all the products were visualized without any sign of smear, indicating that only one sequence size was produced during the PCR. It is important to note that it was possible to species identify all the resulting sequences from both types of bone material, which clearly indicates that DNA is well preserved in the bones after being macerated in the warm water together with Na_2CO_3 and H_2O_2, and when the enzymes, Lipex® 100L and Savinase® 16L Type EX, have been used in the maceration process.

Conclusions

Both maceration techniques showed that it was possible to species identify the collected samples of bone material. What is important when choosing the most optimal maceration technique is that it will quickly provide clean, degreased bones with intact mitochondrial and/or nuclear DNA. Warm-water maceration is a relatively long process (1 week) that also causes an obnoxious smell. The method developed by Simonsen et al. [26] proved to be very efficient concerning time, cleaning and degreasing of bones. Our results also indicate that the mitochondrial DNA is intact when using the above mentioned methods. Our results were without any smeared products opposed to other results that involved the use of chemical maceration methods and all PCR products could be identified to the right species without showing any contamination. The results indicate that the short incubation times and the relatively low heating conditions required when using pure enzymes permit extraction of the mitochondrial DNA present in the bones. This method's long-term effect on DNA was also tested as DNA extraction was not done until two years after the maceration. This indicates that the enzymes do not penetrate into the bone after the maceration is completed.

Acknowledgments

This work was supported by the Research Council of the Danish Ministry of Culture through grant TAKT2007-150. We thank also to Charlotte Hansen, Ole Seeberg and Gitte Petersen for help using the Laboratory of Molecular Systematic at the Natural History Museum of Denmark, and Charlotte Bak (School of Conservation) for help producing chemicals for the agarose gel and Christina Lund for linguistic help.

References

[1] V. Subrahmanyan, J. Duckworth, W. Godden, *The quantitative separation of the skeleton of small animals*, **Biochemical Journal**, **33**, 1939, pp. 1421-1424.

[2] D. Hoffmeister, M. Lee, *Cleaning mammalian skulls with ammonium hydroxide*, **Journal of Mammalogy**, **44**(2), 1963, pp. 283-284.

[3] C.R. Ossian, *Preparation of disarticulated skeletons using enzyme-based laundry "pre-soakers"*, **Copeia**, **1**, 1970, pp. 199-200.

[4] F.C. Hill, *Techniques for skeletonizing vertebrates*, **American Antiquity**, **40**(2), 1975, pp. 215-219.

[5] M. Hounsome, C. Horie, R. Murphy, *Methods of bone preparation*, in **Conservation of Natural History Specimens-Vertebrates,** The Manchester Museum, 1988, p. 19-26.

[6] E. Wet, P. Robertson, I. Plug, *Some techniques for cleaning and degreasing bones and a method for evaluating long-term effects on these techniques*, in **Natural History Collections: Their Management and Value** (Editor: E.M. Herholdt), 1990, p. 37-47.

[7] S. Davis, S. Payne, *101 ways to deal with a dead hedgehog: notes on the preparation of disarticulated skeletons for zoo-archaeological use*, **Circaea**, **8**(2), 1992, pp. 95-104.

[8] H. Sommer, S. Anderson, *Cleaning skeletons with dermestid beetles - two refinements in the method*, **Curator**, **17**(4), 1974, pp. 290-298.

[9] L. Schwartz, **Vertebrate skeletal preparations: A Manual**, The American Biology Teacher, 1977, pp. 35-41.

[10] W.E. Bemis, E.J. Hilton, B. Brown, R. Arrindell, A.M. Richmond, C.D. Little, L. Grande, P.L. Forey, G.J. Nelson, *Methods for preparing dry, partially articulated skeletons of Osteichthyans, with notes on making Ridewood dissections of the cranial skeleton*, **Copeia**, 3, 2004, pp. 603-609.

[11] N. Kurose, R. Masuda, M. Tatara, *Fecal DNA analysis for identifying species and sex of sympatric carnivores: a noninvasive method for conservation on the Tsushima Islands, Japan,* **Journal of Heredity**, **96**(6), 2005, pp. 688-697.

[12] D. Offele, M. Harbeck, R. Dobberstein, N. von Wurmb-Schwark, S. Ritz-Timme, *Soft tissue removal by maceration and feeding of Dermestes sp.: impact on morphological and biomolecular analyses of dental tissues in forensic medicine,* **International Journal of Legal Medicine**, **121**(5), 2007, pp. 341-348.

[13] G.A. Ator, J.C. Andrews, D.S. Maxwell, *Preparation of the human skull for skull base anatomic study,* **Skull Base Surgery**, **3**(1), 1993, pp. 1-6.

[14] L. Yin, S. Venkatesan, S. Kalyanasundaram, Q.H. Qin, *Influence of enzymatic maceration on the microstructure and microhardness of compact bone,* **Biomedical Materials**, **5**(1)**,** 2010, 015006.

[15] J. Arismendi, L. Baker, K. Matteson, *Effects of processing techniques on the forensic DNA analysis of human skeletal remains,* **Journal of Forensic Sciences**, **49**(5), 2004, pp. 930-934.

[16] S.L. Rennick, T.W. Fenton, D.R. Foran, *The effects of skeletal preparation techniques on DNA from human and non-human bone,* **Journal of Forensic Sciences**, **50**(5), 2005, pp. 1016-1019.

[17] D.L.W. Steadman, L. Di Antonio, J. Wilson, K.E. Sheridan, S. Tammariello, *The effects of heat and chemical maceration techniques on the recovery of nuclear an mitochondrial DNA from bone,* **Journal of Forensic Sciences**, **51**(1), 2006, pp. 11-17.

[18] E.J. Lee, J.G. Luedtke, J.L. Allison, C.E. Arber, D.A. Merriwether, D.W. Steadman, *The Effects of Different Maceration Techniques on Nuclear DNA Amplification Using Human Bone,* **Journal of Forensic Sciences**, **55**(4), 2010, pp. 1032-1038.

[19] D.W. von Endt, C.A. Ross, P.E. Hare, *Initial results from cleaning small vertebrate skeletons using the enzyme trypsin,* **Collection Forum**, **13**(2), 1999, pp. 51-62.

[20] T. Fenton, W. Birkby, J. Cornelison, *A fast and safe non-bleaching method for forensic skeletal preparation,* **Journal of Forensic Sciences**, **48**(2), 2003, pp. 274-276.

[21] S. Mairs, B. Swift, G.N. Rutty, *Detergent an alternative approach to traditional bone cleaning methods for forensic practice,* **American Journal of Forensic Medicine and Pathology**, **25**(4), 2004, pp. 276-284.

[22] D.W. Grattan, J. St Hilaire, H.D. Burgess, J.C. McCawley, *The Characterization of Enzymes for Use in Paper Conservation***, Abstracts and Preprints of the International Conference on the Conservation of Library and Archive Materials and the Graphic Arts,** 21—26, September, 1980, Cambridge, Institute for Paper Conservation, Society of Archivists, London, 1980, pp. 21-26.

[23] S.L. Cumbaa, *Osteological preparation techniques used by the zooarchaeological identification center***, Proceedings of 1981 Workshop on Care and Maintenance of Natural History Collections,** (Editor: D.J. Faber), Syllogeus no. 44, National Museums of Canada, Ottawa, 1983, pp. 29-35.

[24] R. Mayden, E. Wiley, *A method of preparing disarticulated skeletons of small fishes,* **Copeia**, **1**, 1984, pp. 230-232.

[25] S.Y. Shelton, J.S. Buckley, *Observations on enzyme preparation effects on skeletal material*, **Collection Forum**, **6**(2), 1990, pp. 76-81.
[26] K.P. Simonsen, A.R. Rasmussen, P. Mathisen, H. Petersen, F. Borup, *A fast preparation of skeletal materials using enzyme maceration*, **Journal of Forensic Sciences**, **56**(2), 2011, pp. 480-484.
[27] M. Margulies, M. Egholm, W.E. Altman, S. Attiya, J.S. Bader, L.A. Bemben, J. Berka, …, J.M. Rothberg, *Genome sequencing in microfabricated high-density picolitre reactors*, **Nature**, **437**(7057), 2005, pp. 376-380.
[28] E. Willerslev, A. Cooper, *Ancient DNA*, **Proceedings of the Royal Society B: Biological Sciences**, **272**(1558), 2005, pp. 3-16.
[29] W. Miller, D.I. Drautz, J.E. Janecka, A.M. Lesk, A. Ratan, L.P. Tomsho, M. Packard, …, S.C. Schuster, *The mitochondrial genome sequence of the Tasmanian tiger (Thylacinus cynocephalus)*, **Genome Research**, **19**(2), 2009, pp. 213-220.
[30] M. Rasmussen, Y. Li, S. Lindgreen, J. Pedersen, A. Albrechtsen, I. Moltke, M. Metspalu, …, E. Willerslev, *Ancient human genome sequence of an extinct Palaeo-Eskimo*, **Nature**, **463**(7282), 2010, pp. 757-762.
[31] A. Davison, J.D.S. Birks, R.C. Brookes, T.C. Braithwaaite, J.E. Messenger, *On the origin of faeces: morphological versus molecular methods for surveying rare carnivores from their scats*, **Journal of Zoological London**, **257**, 2002, pp. 141-143.
[32] D.W. Steadman, L.L. DiAntonio, J.J. Wilson, K.E. Sheridan, S.P. Tammariello, *The Effects of Chemical and Heat Maceration Techniques on the Recovery of Nuclear and Mitochondrial DNA from Bone*, **Journal of Forensic Sciences**, **51**(1), 2005, pp. 11-17.

Received: September 10, 2012
Accepted: March, 18, 2013