Gene, 167 (1995) 327–332

GENE 09401

Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles

(Ancient DNA; molecular anthropology; single-copy gene; polymerase chain reaction; gender determination)

Marina Faerman[a], Dvora Filon[b], Gila Kahila[a], Charles L. Greenblatt[c], Patricia Smith[a] and Ariella Oppenheim[b]

[a]*Dental Division of Anatomy, Tel. (972-2) 758-577, Hebrew University, Hadassah Faculty of Dental Medicine, and Departments of* [b]*Hematology and* [c]*Parasitology, Hebrew University, Hadassah Medical School and Hadassah University Hospital, Jerusalem 91120, Israel. Tel. (972-2) 758-081*

Received by S. Woodward: 4 May 1995; Accepted: 12 July 1995; Received at publishers: 28 September 1995

SUMMARY

Sex identification of archaeological human remains is essential for the exploration of gender differences in past populations. Traditional morphometric analyses fail to identify the gender of incomplete skeletal remains and that of immature individuals. In the present work, we have established a sensitive and reliable method, based on amplification of the single-copy amelogenin-encoding gene (*AMG*). The Y allele carries a small deletion in the first intron, facilitating the design of distinct X- and Y-specific polymerase chain reactions. Amplification with three primers, two of which are allele-specific, allows unambiguous identification of both X and Y chromosome signals in a single reaction, providing an internal control. For added confidence, the reaction may be performed in separate tubes for each allele. Using this method, the sex was determined from the skeletal remains of 18 individuals, including young children, out of 22 examined from periods ranging from 200 to around 8000 years ago. The state of skeletal preservation ranged from poor to good. Cortical and cranial bones, as well as teeth, were found to provide sufficiently preserved DNA. The success of retrieval of amplifiable DNA was not related either to the period or to the burial site. On the other hand, the method of DNA purification was critical. In our hands, direct DNA purification by Chelex from minute samples of bone/tooth powder gave the best results. This study demonstrates the applicability of the method for gender determination in skeletal remains from different periods.

INTRODUCTION

Investigation of gender differences plays an important role in the reconstruction of the social structure of past societies. Gender has been traditionally determined through the identification of grave goods and by bone morphometric analyses. However, for fragmentary adult skeletons or those of children and infants conventional anthropometric methods are unreliable. Analyses of DNA sequences specific to the X and/or Y chromosomes may provide an ideal solution.

There are still many difficulties and methodological problems in analyzing DNA obtained from archaeological specimens. PCR is extremely sensitive and is therefore prone to contamination by non-relevant human DNA material both in the field and in the laboratory. Other problems are the highly degraded state of ancient DNA and presence of inhibitors of unknown origin which interfere with the PCR reaction (Paabo et al., 1988; Hagelberg et al., 1991).

Correspondence to: Dr. A. Oppenheim, Department of Hematology, Hebrew University, Hadassah Medical School, Jerusalem 91120, Israel. Tel. (972-2) 776-753; Fax (972-2) 423-067; e-mail: Ariella@md2huji.ac.il

Abbreviations: *AMG*, amelogenin-encoding gene; bp, base pair(s); BP, before present; BSA, bovine serum albumin; EtdBr, ethidium bromide; kb, kilobase(s) or 1000 bp; PCR, polymerase chain reaction; u, unit(s).

SSDI 0378-1119(95)00697-4

DNA-based sex determination of skeletal remains was first performed by amplification of Y chromosome-specific sequences (Hummel and Herrmann, 1991). However, negative findings, usually interpreted as a female, may also result from poor preservation of DNA. Amplification of the divergent X and Y alleles of the *AMG* gene offers the advantage of an internal positive control (Akane et al., 1991). The Y allele carries several sequence variations in the first intron, including a deletion of 189 bp (Nakahori et al., 1991). However, this method requires relatively large amounts of non-degraded DNA. For ancient DNA, a PCR reaction based on amplifying a smaller section of the *AMG* gene is more suitable (Sullivan et al., 1993).

We have developed a highly sensitive reaction based on amplifying part of intron 1 of the *AMG* gene, using a strategy employing three primers, two of which are allele-specific. The method facilitates analysis of samples with degraded DNA, such as frequently encountered in archaeological specimens, with high confidence.

EXPERIMENTAL AND DISCUSSION

(a) Development of PCR analysis for sex determination

As there are few reports on analyses of single copy genes in archaeological specimens, a major aim of this study was to determine the reliability of the method and to investigate several parameters which may influence the analysis. We compared the source of the DNA (tooth versus several types of bones) and methods of DNA extraction. In particular, it was of interest to determine whether one can predict the possibility of successful DNA analyses by examining the gross state of bone preservation.

The strategy developed for sex determination in skeletal remains is described in Fig. 1. In order to amplify the highly degraded DNA, usually found in archaeological specimens, the primers span short DNA fragments from the *AMG* sequence (Nakahori et al., 1991). For added specificity three primers are employed: the 5′-primer (M4, 5′-CAGCTTCCCAGTTTAAGCTCT) is common to both the X and Y *AMG* alleles, while the 3′-primers are specific for one or the other. The 3′ X-specific primer (M5, 5′-TCTCCTATACCACTTAGTCACT) is derived from the sequence deleted in the Y chromosome and the 3′ Y-specific primer (M6, 5′-GCCCAAAGTTAGTA-ATTTTACCT) covers the joint of the deletion break-points. The 330-bp and 218-bp PCR products, from the X and Y chromosomes, respectively, are detected by agarose gel electrophoresis. Usually the three primers are used in a single tube reaction. However, in ambiguous cases, the X and Y-specific alleles can be amplified in separate tubes, providing an additional factor of confidence.

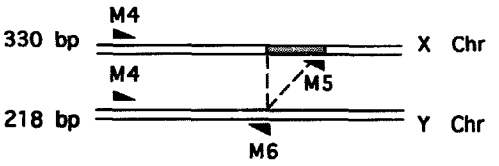

Fig. 1. Development of PCR analysis for gender determination. Part of the *AMG* gene showing the location of the PCR primers and the size of the products. Fragment deleted in the Y chromosome (Chr) is blackened.

Conditions for the PCR reaction were established using contemporary DNA. Our experiments demonstrated very high sensitivity. The level of detection for female DNA was 25 pg and for male DNA 5–10 pg, which is approximately equivalent to DNA obtained from a single diploid cell.

(b) Reliability of the DNA test for sex determination in skeletal remains

The skeletal remains analyzed are listed in Tables I and II. All were found in well defined archaeological context from sites all over Israel. The specimens came from a submerged Neolithic site, from four sandy coastal sites (Coastal A, B, C and D), from limestone caves in the desert (Desert A and B) and from an earth filled cistern in a limestone ridge (Mountain).

First we studied four specimens (Nos. 1–4, Table I, three males and one female) whose gender was known from morphological parameters. DNA was isolated by decalcification of bone powder followed by proteinase K and phenol-chloroform purification (Hagelberg and Clegg, 1991). Only the male specimen (No. 2, Table I) gave a 218-bp amplification product corresponding to the Y-*AMG* allele (not shown). Failure to amplify DNA may result from the low amount and degraded state of DNA in archaeological samples and from the presence of inhibitors which interfere with the PCR reaction (Paabo et al., 1988; Hagelberg et al., 1991). Repurification by treatment with Chelex 100 (Akane et al., 1993) greatly improved the results (Fig. 2A and Table I, specimens Nos. 1, 3, 4). To confirm that the single (218-bp) band seen in specimen No. 1 represents the Y allele and not some nonspecific amplification, the DNA was amplified with X-specific and Y-specific primers in two separate tubes. The results (Fig. 2B) demonstrate amplification only with the Y-specific primers, and not with the X-specific ones.

The high sensitivity of PCR is a source of a great number of artefacts. To verify authenticity of the X- and Y-specific PCR products, the respective bands of specimen No. 4 (Fig. 2A) were subjected to sequence analysis in parallel to DNA extracted from a tooth of the same individual and to contemporary DNA control (Fig. 3).

TABLE I

Gender determination of various skeletal remains using different methods of DNA purification

Site[a]	Date (years BP)[b]	Gender (bone morphology)[c]	Tissue sampled[d]	DNA extraction[e]	Gender (DNA analysis)[f]
1. Submerged-1[g]	8100	male	femur	phenol	undetermined
			femur	phenol+Chelex	male[h]
			rib	Chelex	undetermined
2. Coastal A-1	300	male	femur	phenol	male[h]
				phenol+Chelex	male[h]
3. Coastal A-2	300	female	humerus	phenol	undetermined
				phenol+Chelex	female
			tooth-M2	Chelex	female
4. Coastal A-3	300	male	tibia	phenol	undetermined
				phenol+Chelex	male
			tooth-M1	Chelex	male
5. Coastal B-1	200	female	femur	phenol+Chelex	undetermined
				Chelex	undetermined
6. Coastal B-2	200	female	femur	phenol+Chelex	undetermined
				Chelex	female
7. Desert A	2400	female	tooth-M2	Chelex	female
8. Desert B	2400	unknown (child)	skull	phenol	male[h]
				Chelex	male
9. Coastal A-4	300	unknown (incomplete)	skull	phenol	undetermined
				phenol+Chelex	male[h]
10. Submerged-2	8100	unknown (incomplete)	tibia	phenol	undetermined
			tibia	phenol+Chelex	male[h]
11. Costal B-3	300	unknown (child)	femur	phenol	undetermined
				phenol+Chelex	undetermined
				Chelex	male

[a] Description of the archaeological site.
[b] Date of the specimen in years before present (BP).
[c] Sex of the individual as determined by morphometric analyses.
[d] Tissue sampled for DNA analyses.
[e] Method of DNA purification.
[f] Sex of the individual as determined by DNA analyses.
[g] This specimen was described by Herskovitz et al. (1991).
[h] Only the lower band corresponding to the Y chromosome was amplified.

TABLE II

Gender determination in DNA samples purified with Chelex[a]

Site	Date (years BP)	Tissue sampled	Successful PCR reactions[b]	Gender
12. Mountain-1	2400	tooth-M1	5/6	male
13. Mountain-2	2400	ulna	5/6	male
14. Mountain-3	2400	ulna	0/6	undetermined
15. Mountain-4	2400	rib	0/6	undetermined
		tooth-M2	1/6	male[c]
16. Coastal C-1	2000	femur	6/7	male
17. Coastal C-2	2000	femur	2/6	female
18. Coastal D-1	2700	humerus	0/6	undetermined
19. Coastal D-2	2700	humerus	0/6	undetermined
20. Coastal D-3	2700	humerus	2/6	male
21. Coastal D-4	2700	humerus	2/6	male[c]
22. Coastal D-5	2700	humerus	2/6	male

[a] See Table I.
[b] Number of successful PCR reactions out of total reactions performed.
[c] Only the lower band corresponding to the Y chromosome was amplified.

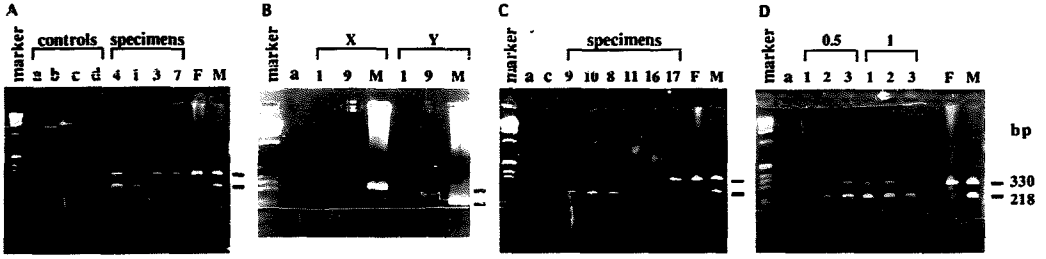

Fig. 2. Reliability of the DNA test for gender determination in skeletal remains. **(A)** Analyses of specimens obtained from skeletal remains of known gender as previously determined from the morphometric studies. Marker was '1 kb DNA ladder' (BRL); controls: a, 'no DNA' PCR control; b, 'blank extraction' control for DNA extracted with phenol and analyzed in a different gel (not shown); c, 'blank extraction' Chelex – control for specimen No. 7; d, 'blank extraction' control for phenol-extracted DNA repurified with Chelex – control for specimens Nos. 4, 1, 3; specimen numbers are as in Tables I and II. F, M, contemporary female and male DNA, respectively. **(B)** Separate amplification of the X and Y alleles. DNA of specimens No. 1 and No. 9 was amplified with the X-specific primers M4 and M5 and with the Y-specific primers M4 and M6 in two separate tubes, as designated at the top. Marker and controls are as in panel A. **(C)** DNA-based gender identification of skeletal remains of unknown gender. **(D)** Dependence of the PCR reaction on the amount of DNA extract. DNA was extracted from approximately 0.5 mg and 1 mg (as designated) of bone powder obtained from specimen No. 12; PCR with 3, 7 and 11 µl of each of the DNA extracts are shown under 1, 2 and 3, respectively. Marker and controls are as in panel A. 'Blank extraction' Chelex control of this experiment is shown in panel C, lane c. Methods. DNA was isolated from various parts of the skeleton including the skull, ulna, femur, tibia, rib and also teeth, using two methods. The first was phenol-chloroform extraction (Hagelberg and Clegg, 1991). We have added a step of extensive dialysis (in the cold against TE (10 mM Tris·HCl/1 mM EDTA, pH 7.4)) before concentrating the DNA extract through Centricon-30 microconcentrators. The second method for DNA purification was by Chelex 100 (Woodward et al., 1994). Some of the DNA samples extracted by the phenol-chloroform procedure (50 µl) were re-purified by Chelex 100 (500 µl). At least two independent DNA extractions from approx. 0.5 mg and approx. 1 mg of bone powder were performed for each bone specimen, in parallel to a 'blank extraction' control (containing no bone material). Extreme care was undertaken to avoid contamination with contemporary human DNA as previously described (Filon et al., 1995). The bone samples were handled (using masks, gloves and laboratory coats) in a separate location, where no DNA analyses are performed. Extraction and the setting up of the PCR reactions of ancient DNA were performed in two separate hoods with daily irradiated UV (in different rooms), physically separated from the analyses of the PCR products and from contemporary DNA work. Disposable sterile tubes, filtered tips, and aliquoted sterile reagents and solutions (prepared with injection-grade H_2O), exclusively dedicated to ancient DNA studies, were used throughout. Different sets of pipettes were used for DNA extraction, PCR amplification and for analysis of the PCR products. Three different amounts (3, 7 and 11 µl) of each ancient DNA extract were subjected to PCR along with appropriate controls. 'Hot-start' PCR was performed in 25 µl reactions using 1.25 u Taq polymerase (Appligene) and the original buffer/18 ng primer M4/9 ng each of M5 and M6/BSA (40 ng/reaction)/2.5 mM $MgCl_2$ for 40 cycles: 1 min at 94°C, 2 min at 50°C and 2 min at 72°C. 18 µl aliquots were analyzed on 2% Nusieve agarose gels, and visualized by EtdBr staining.

To avoid cross contamination, the three DNA samples were extracted and amplified separately. The three sequences were identical to the published *AMG* sequence (Nakahori et al., 1991).

Direct DNA purification by Chelex in a single step significantly improved the results (Table I, Nos. 3, 4, 6–8, 11). An added virtue of the Chelex method is its applicability to minute samples (less than a milligram) of bone/tooth powder, thus minimizing damage to the specimen. We have therefore adopted a strategy whereby we prepare small DNA samples, sufficient for just a few analyses. An additional advantage is that there is no need to store ancient DNA over extended time periods. In fact, the DNA for future studies is probably best 'stored' in the bones.

Several lines of evidence suggest that in our PCR assay the Y allele is amplified more readily than the X allele. In contemporary DNA (data not shown), the Y-specific band was still visible when the DNA was diluted to 5 pg/reaction, while the X-specific band in both male and female samples was seen only at 25 pg/reaction and above. As seen in Tables I and II some of the ancient male samples reproducibly yielded the Y band only. Furthermore, Fig. 2D shows preferential amplification of the Y allele as the reaction conditions became less favorable. The higher sensitivity of detection of the Y allele may be due to the shorter amplification product or to the nature of the allele-specific primers. Regardless of the reason, this suggests that our method is more prone to miss a female specimen than a male. On the other hand, the more efficient amplification of the Y allele suggests that presence of a single 330-bp product indicates a female, and is not a false negative result of a male.

Altogether, seven adult specimens whose gender had been previously determined through morphometric characteristics were analyzed. For six of these the DNA analyses confirmed the original gender identification. For the seventh (No. 5), no amplification products were obtained. In no case was there a discrepancy between the DNA and the morphometric studies.

(c) Optimizing the amount of DNA in the PCR reaction

We considered the amount of DNA extract in the PCR reaction an important factor, since increasing the amount

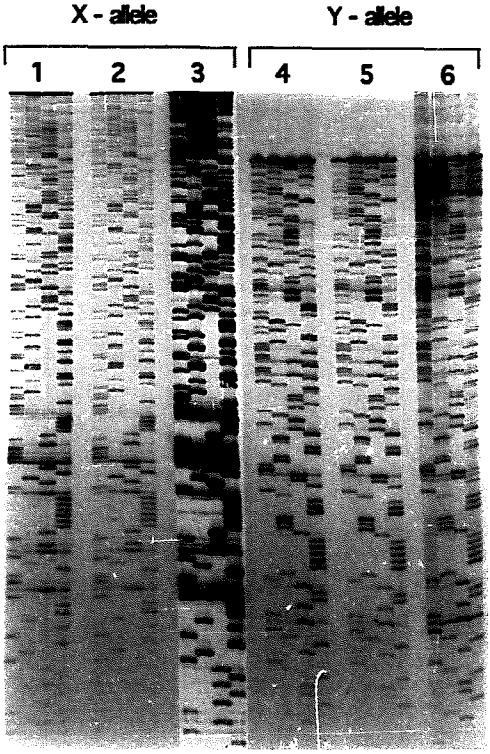

Fig. 3. Sequence analyses of the PCR products. PCR products of DNA purified from tibia and tooth (M1) of specimen No. 4 were separated on a low melting agarose gel. The X and Y-specific bands were cut off the gel and kept frozen. Before sequencing, the agarose was melted at 68°C and 14 µl were used for each reaction. Sequencing was performed, using the allele-specific M5 and M6 primers, with Sequenase Version 2 (US Biochemical, Cleveland, OH, USA), using 8 M urea-6% polyacrylamide gel). Lanes 1 and 4, DNA extracted from tibia; lanes 2 and 5, tooth; lanes 3 and 6, contemporary DNA control.

of DNA, which was likely to improve the reaction, entailed a parallel increase in the level of inhibitors. Fig. 2D demonstrates amplification of different amounts of DNA of the same ancient specimen (No. 12) obtained from two extractions carried out in parallel, using approximately 0.5 and 1 mg of bone powder. No PCR products are seen either when too little or too much DNA extract was used. In other experiments, we have obtained results only either at the lowest or at the highest amounts of DNA extracts analyzed.

These results demonstrate the importance of analyzing several amounts of DNA extracts for each archaeological specimen. We therefore adopted a routine procedure of six PCR reactions from two independent extractions of different amounts of bone powder, for each specimen. Table II lists the number of successful PCR reactions for each specimen. It can be seen that it varies widely from sample to sample.

(d) Sex determination of skeletal remains obtained from various archaeological sites and periods

First, 4 specimens whose gender was unknown (Nos. 8–11) were analyzed. Two of these were children (Nos. 8, 11), and the skeletal remains of the other two were incomplete. Specimen No. 9 included only fragmentary cranial bones and No. 10, fragmentary tibia. Sex was determined for all four samples, as seen in Fig. 2C and Table I. Again, only one of the DNA samples gave PCR products after the phenol extraction. Chelex purification facilitated PCR amplification of the other three DNA samples.

Studies on eleven additional specimens, whose gender could not be previously identified, either due to their young age or because the skeletal remains were fragmentary (Nos. 12–22, Table II), substantiated that Chelex extraction is satisfactory in most cases. Only four specimens of the total 22 studied did not yield results. For specimen No. 5, 200 years old, no PCR products were seen in nine reactions (three reactions of the single phenol+Chelex extraction and six reactions of two Chelex extractions). Specimens Nos. 14, 18 and 19, 2400 and 2700 years old, did not give any PCR products in a total of six reactions for each specimen (two DNA extractions and three concentrations each). In three of the specimens DNA was prepared from both bones and teeth. In all three the results obtained from both sources were consistent, demonstrating the reliability of the method.

(e) Success of DNA retrieval in relation to the tissue sampled, date and local conditions of the burial

Long cortical bones (such as femur, tibia, humerus, ulna), cranial bones (Nos. 5, 6) and dental pulp (Nos. 3, 4, 7, 15) appeared to be a good source for ancient DNA, while rib samples (Nos. 1, 15) did not. Although the amount of DNA in a single tooth is very small, all four teeth tested yielded amplifiable DNA. The general gross appearance of the bone was not indicative of the state of DNA preservation, in agreement with others (Hagelberg et al., 1991). For example, DNA preservation in brittle and readily crumbled bones from Coastal A site was better than in complete, dense and yellowish specimens from Coastal B site dated to the same period.

Comparison of the various sites and periods indicates that in general, success of DNA retrieval was not related to either, as previously suggested (Hagelberg et al., 1989). For example, DNA preservation in the specimens obtained from coastal sites, all in sandy soil, varied widely. Some of the older specimens (Nos. 16, 17, 20–22)

were better preserved than the more recent ones (Nos. 8–10). Most striking is the relatively good DNA preservation in the 8100 years old specimens excavated from a submerged Neolithic site (Nos. 1, 10). Specimens from the Israeli desert sites were also found to be satisfactorily preserved (Nos. 7, 8).

Most interestingly, we frequently observed variation in DNA preservation in specimens obtained from different burials within the same archaeological site (Nos. 12–15). Surprisingly, even within the same burial (Nos. 18–22) DNA was not equally preserved. Moreover, in another study (Filon et al., 1995) we observed that the state of DNA preservation in different bones of the same individual, or even parts of the same bone, may vary widely. It appears that preservation is influenced to a far greater extent by the local conditions of the burial, probably the type of soil, humidity and aerobic conditions. These findings indicate that not only the general macroenvironment of the burial, but also microenvironment of the specific bone, is critical for DNA preservation.

(f) Conclusions

(*1*) The method of selective amplification of the X and Y *AMG* alleles by three primers is highly sensitive and reliable. There was no discrepancy between the results of the DNA tests and the morphometric analyses. Furthermore, sex was successfully determined on 12 of 15 fragmentary skeletal remains, whose gender was unknown. Thus, this method provides an important tool for sex identification in forensic medicine and for examination of gender differences in past societies.

(*2*) Direct DNA purification by Chelex is superior to the other protocols examined here and allows purification of amplifiable DNA from minute samples, minimizing damage to precious ancient specimens.

(*3*) The precise amount of DNA extract in the PCR reaction is highly important, presumably providing optimal balance between DNA and inhibitors.

(*4*) DNA is better preserved in teeth, cranial and long cortical bones than in ribs.

(*5*) General appearance of the bones is not indicative of the state of DNA preservation.

(*6*) Success of DNA retrieval is not related either to the archaeological site or to its date. DNA preservation is greatly influenced by the local conditions and microenvironment of the specimen in question.

ACKNOWLEDGEMENTS

We wish to thank Dr. Israel Hershkovitz for providing the Neolithic samples. This research was supported by The Israel Science Foundation, administered by The Israel Academy of Sciences and Humanities, and by The Hebrew University Internal Funds.

REFERENCES

Akane, A., Shiono, H., Matsubara, K., Nakahori, Y., Seki, S., Nagafuchi, S., Yamada, M. and Nakagome, Y.: Sex identification of forensic specimens by polymerase chain reaction (PCR): two alternative methods. Forensic Sci. Int. 49 (1991) 81–88.

Akane, A., Shiono, H., Matsubara, K., Nakamura, H., Hasegawa, M. and Kagawa, M.: Purification of forensic specimens for the polymerase chain reaction (PCR) analysis. J. Forensic Sci. 38 (1993) 691–701.

Filon, D., Faerman, M., Smith, P. and Oppenheim, A.: Sequence analysis reveals a β-thalassemia mutation in the DNA of skeletal remains from the archaeological site of Akhziv, Israel. Nature Genet. 9 (1995) 365–368.

Hagelberg, E., Sykes, B. and Hedges, R.: Ancient bone DNA amplified. Nature 342 (1989) 485.

Hagelberg, E. and Clegg, J.B.: Isolation and characterization of DNA from archaeological bone. Proc. R. Soc. London B 244 (1991) 45–50.

Hagelberg, E., Bell, L.S., Allen, T., Boyde, A., Jones, S. and Clegg, J.: Analysis of ancient bone DNA: techniques and applications. Philos. Trans. R. Soc. London B 333 (1991) 399–407.

Hershkovitz, I., Ring, B., Speirs, M., Galili, E., Kislev, M., Edelson, G. and Hershkovitz, A.: Possible congenital hemolytic anemia in prehistoric coastal inhabitants of Israel. Am. J. Phys Anthropol. 85 (1991) 7–13.

Hummel, S. and Herrmann, B.: Y-Chromosome-specific DNA amplified in ancient human bone. Naturwissenschaften 78 (1991) 266–267.

Nakahori, Y., Takenaka, O. and Nakagome, Y.: A human X-Y homologous region encodes 'amelogenin'. Genomics 9 (1991) 264–269.

Paabo, S., Gifford, J.A. and Wilson, A.C.: Mitochondrial DNA sequences from a 7000-year-old brain. Nucleic Acids Res. 16 (1988) 9775–9787.

Sullivan, K.M., Mannucci, A., Kimpton, C.P. and Gill, P.: A rapid and quantitative DNA gender test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. Biotechniques 15 (1993) 636–641.

Woodward, S.R., King, M.J., Chiu, N.M., Kuchar, M.J. and Griggs, C.W.: Amplification of ancient nuclear DNA from teeth and soft tissues. PCR Methods Appl. 3 (1994) 244–247.