Sequence analysis reveals a β-thalassaemia mutation in the DNA of skeletal remains from the archaeological site of Akhziv, Israel

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β-Thalassaemia is manifested by severe anaemia and extensive bone pathology. Similar pathology may also result from other forms of anaemia. To clarify the precise cause, we performed DNA analyses on archaeological remains of a child with severe bone pathology. We found homozygosity for frameshift in codon 8 of β-globin, causing a β-null phenotype. Paradoxically, the child died when eight years old, whereas such patients are transfusion dependent from early infancy. An infrequent polymorphic marker in the child's DNA, and information from present-day patients, indicated that amelioration of the clinical condition was due to elevated fetal haemoglobin production. Thus this analysis provided not only precise diagnosis of a genetic disease but also allowed clarification of the molecular mechanism underlying the clinical presentation.

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β-Thalassaemia major is a severe hereditary anaemia caused by any of approximately 150 mutations in the β-globin gene. Without frequent blood transfusions the disease is lethal in early childhood. The high prevalence of this autosomal recessive disease in malaria-infested regions led Haldane[1] to propose that heterozygous carriers have a selective advantage in a malarial environment. Angel[2] suggested that the disease is ancient, providing genetic protection in the heterozygous state against the increased prevalence of malaria that followed the establishment of permanent settlements near standing water in the Neolithic period. Until now the evolution of the disease has been studied from two perspectives. The first is extrapolation from the distribution of present day mutations in different modern populations[3–5]. The second is through attempts to identify the disease in skeletal remains on the basis of bone pathology.

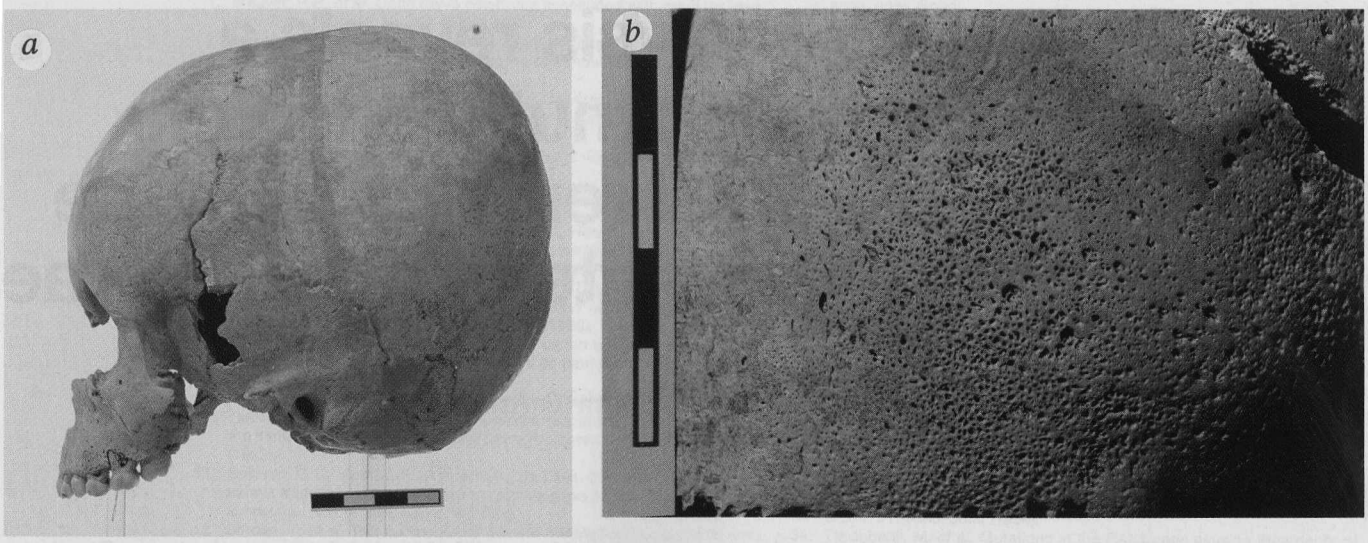
The severe anaemia and increased spatial demands for the hyperplastic erythropoietic marrow cause extensive skeletal pathology, which is most pronounced in the skull and characterized by diploic thickening and "hair on end" appearance[6]. Such pathology in archaeological specimens has been attributed to thalassaemia[2]. It has also been suggested that anaemia from other causes as well as malnutrition may cause similar effects[7]. The differential diagnosis is important both for tracing the evolution and spread of this disease and for understanding environmental pressures experienced by past populations. Recent developments in DNA technology provide the opportunity for probing this question. Using this approach we identified a β-thalassaemia mutation in skeletal remains of a child, excavated from an archaeological site at Akhziv, on the Northern coast of Israel.

The archaeological site

Tel Akhziv, located 15 km north of Acre, has been inhabited with only slight interruptions for at least 4,000 years. The region was infested by malaria until the beginning of this century. Four distinct cemetery complexes have been identified in the vicinity of the Tel. At least two of those have been dated to the Phoenician period, 11th to 7th centuries B.C.E.[8]. While excavating the Northern Phoenician cemetery, a number of unmarked graves were found. They had been dug into the Phoenican cemetery and themselves disturbed by later burials. The skeletal remains were identified as Moslem by their position. They were lying on their backs in an east-west orientation with the head facing south towards Mecca. Coins found in some of the graves indicated that they dated to the Ottoman period, sometime between the 16th and 19th centuries. The skull of the child studied here was recovered from one of the disturbed Ottoman graves, containing the remains of four individuals. The disturbed graves probably belong to the earlier part of the period. Since ^{14}C variations in the atmosphere within that time period do not allow more precise calendrical determination (R. Hedges, personal communication), carbon dating was not performed.

Bone pathology

The skull showed severe bone pathology known as porotic hyperostosis (Fig. 1). Pathological features include extensive pitting of the posterior portion of the skull (parietal and occipital bones, Fig. 1*b*), mild pitting of the frontal bone and pitting of the orbital roof (cribra orbitalia, Fig. 1*c*). The cranial bones are thick with a maximum of 10 mm in the parietal region (Fig. 1*d*), compared with an average thickness of 4.5 mm for individuals of the same



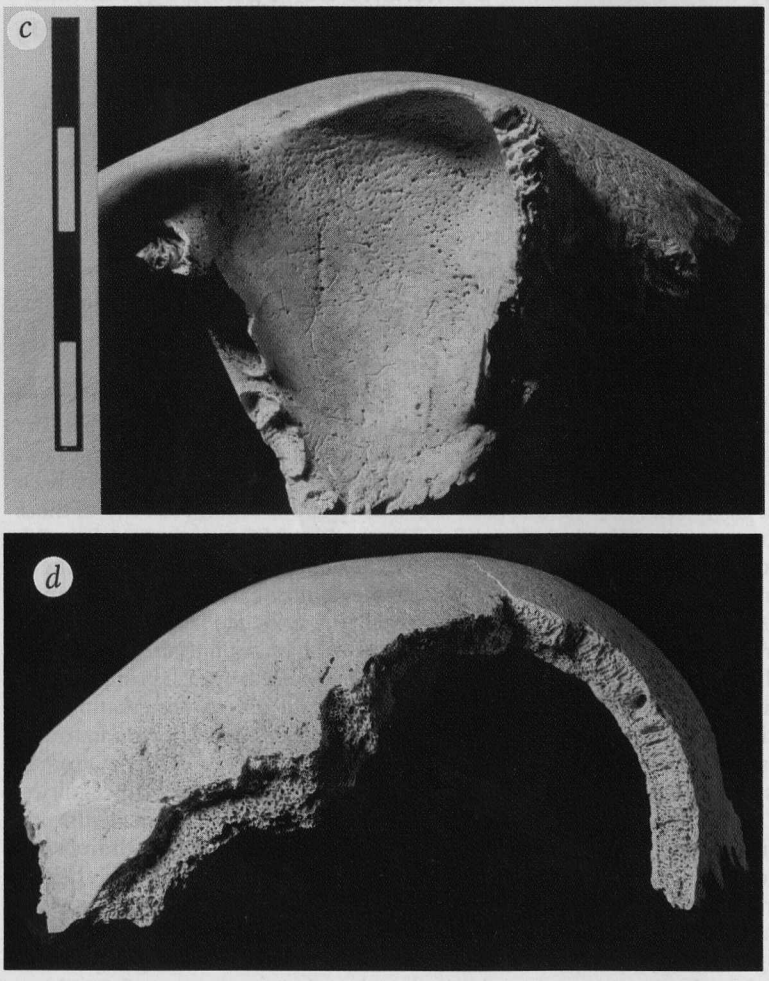


Fig. 1 Skull of child of Akhziv. *a*, Lateral view of the reconstructed skull. *b*, Outer surface of the parietal bone showing extensive pitting. The scale bar is in centimeters. *c*, Detail of the orbital roof showing a mild degree of pitting (cribra orbitalia). *d*, Broken section of the parietal bone before reconstruction. The broken surface shows thickening of the diploë and honeycombed compartments of irregular subperiosteal bone formation replacing the outer plate.

age and period[9]. Computerized tomography shows the same pattern of pathology in other regions of the skull (Fig. 2). Honeycombed compartments of irregular subperiosteal bone formation are seen at the broken section (Fig. 1*d*). This combination of pathological signs in young children is considered indicative of thalassaemia major[2]. However, the stage of dental development suggested an age of seven to eight years, indicating a clinical course that was not very severe. We therefore sought evidence at the DNA level.

DNA studies

There are still many difficulties and methodological problems in analysing DNA obtained from archaeological specimens. The technology of polymerase chain reaction (PCR) is extremely sensitive and is therefore prone to contamination by non-relevant human DNA material, such as from personnel handling the specimens in the field or in the laboratory. Another problem is the presence, in ancient DNA extracts, of inhibitors of unknown origin that interfere with the PCR reaction[10]. In our hands, purification by the chelating ion-exchange resin Chelex[11] yielded best results.

Elaborate precautions were undertaken to avoid contamination by non-relevant DNA (see Methodology). We have found that the amount of DNA in the PCR reaction is critical, presumably because of the inhibitors. Therefore, six different amounts of DNA (from two independent DNA extractions) were used for PCR, along with appropriate controls. For a positive control we used contemporary DNA of the person who handled the ancient DNA samples. After 40 amplification cycles (annealing temperature was 50 °C) only one of the six samples showed a faint DNA band, which was further amplified (re-PCR) to obtain sufficient amount of DNA for sequence analysis. Sequencing of the noncoding strand (Fig. 3*a*) demonstrated homozygosity for a frameshift mutation in codon 8 (FS8) due to a TT deletion (AA in the coding strand). This was confirmed by analysis of the coding strand (Fig. 3*b*). In addition, Fig. 3*a*,*b* shows a rare DNA polymorphism, C→T in the second codon of β-globin, with no alteration in the amino acid. This polymorphism is present in 13% of the Mediterranean β-thalassaemia chromosomes[12].

Because of the many artefacts in ancient DNA analysis, PCR was performed on additional DNA samples prepared

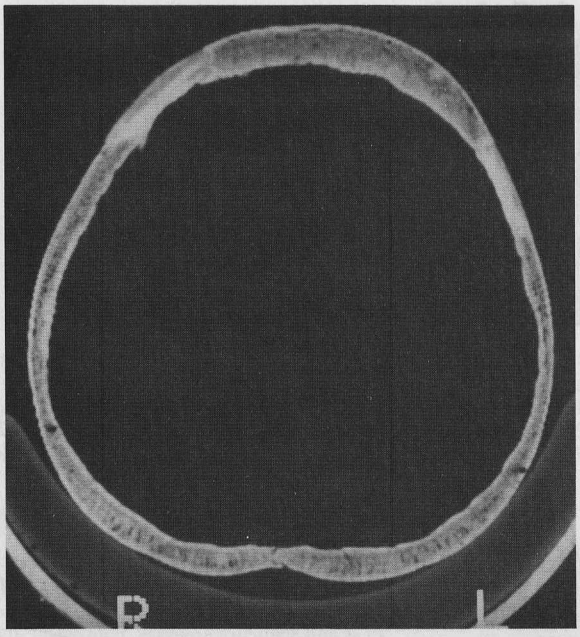


Fig. 2 Computerized tomography of the skull. This view, taken through the horizontal plane, shows diploic thickening of the frontal and parietal bones.

from two other bone fragments of the same skull. In this experiment the annealing temperature was lowered from 50 °C to 48 °C. One of the fragments did not yield any β-globin amplifiable DNA. The other yielded PCR products, of different intensities, in all six tubes (Fig. 4*a*). Nonspecific fragments were seen in addition to the fragment of the expected size, presumably because of the lower annealing temperature. Southern blot analysis with FS8-specific oligonucleotide probes (mutant and normal) demonstrated the presence of the mutation in all six 232-bp reaction products (some very faint, Fig. 4*b*). These results, and the presence of the codon 2 C→T polymorphism, were further confirmed by sequence analyses (not shown) of two of the PCR products (lanes 3 and 6, Fig. 4*a*), obtained from two different DNA preparations.

Discussion

Most studies on ancient DNA focus on analyses of mitochondrial DNA, due to its high copy number in eukaryotic cells. In the present work we have identified a mutation in β-globin, a single-copy gene. This finding allows tracing the FS8 mutant allele in Israel back to the Ottoman period. Further studies, on older skeletal remains, will help to clarify mechanisms in the evolution of thalassaemia in this region.

It may be of significance that the three bone fragments of the same skull differed greatly in the state of DNA preservation. The first gave only a faint band in one of six PCR reactions. No amplifiable DNA was obtained from the second fragment, while the third yielded β-globin PCR products in all six reactions, although the second and the third bone fragments were analysed in parallel. Therefore it appears that the state of DNA preservation in different bones of the same individual, or even parts of the same bone, may vary widely. Further research is needed to clarify this issue.

The mutation FS8 is spread at a frequency of 2 to 10% of all thalassaemia genes throughout the Eastern Mediterranean basin. The highest frequency (10%) has been reported from Turkey[13]. In present-day Israel the mutation is found almost exclusively in Arabs, with a frequency of 2.3%[14]. The mutant allele may have been brought to Israel from Turkey. The homozygosity of the child for a single, uncommon mutation suggests consanguineous marriage, as widely practiced among Israeli Moslems.

The frameshift mutation leads to a β-globin null phenotype (β°-thalassaemia). Patients with β°-thalassaemia are usually transfusion dependent from an early age. During fetal life and early infancy they survive by virtue of their fetal haemoglobin ($\alpha_2\gamma_2$), which is normally replaced by adult haemoglobin ($\alpha_2\beta_2$) during the first year of life. Without transfusions, children with β°-thalassaemia succumb in early childhood. Paradoxically, the child investigated here died at an estimated age of eight years.

Some β-thalassaemia patients continue to produce fetal haemoglobin at significant levels, which ameliorates the clinical course. This condition is usually associated either with specific γ-globin promoter mutations, with large deletions in the β-globin gene cluster, or (for as yet unknown reason) with certain Mediterranean β-globin cluster haplotypes (III, IV and IX, as classified by Orkin *et al.*[12]). These haplotypes share a common pattern of polymorphic markers in the 5' region of the β-globin gene cluster encompassing ε, γ and ψβ-globin genes. Usually, β-thalassaemia mutations are found linked to specific haplotypes, suggesting that the mutational events have occurred on specific haplotypic backgrounds[12].

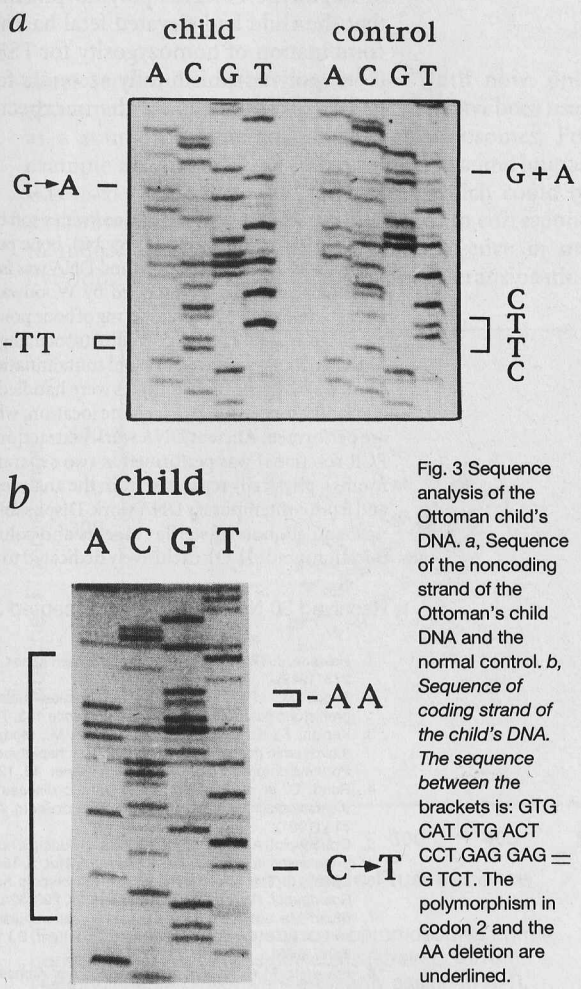


Fig. 3 Sequence analysis of the Ottoman child's DNA. *a*, Sequence of the noncoding strand of the Ottoman's child DNA and the normal control. *b*, *Sequence of coding strand of the child's DNA. The sequence between the* brackets is: GTG CAT CTG ACT CCT GAG GAG = G TCT. The polymorphism in codon 2 and the AA deletion are underlined.

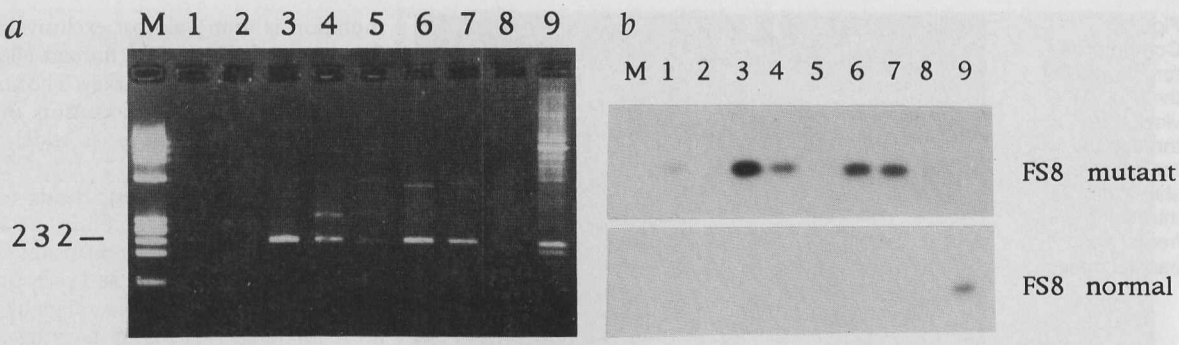


Fig. 4 Identification of the FS8 mutation in PCR products of the Ottoman child's DNA. DNA obtained from the third cranial fragment was analysed in parallel to the first DNA preparation. The PCR products (18 μl) were separated on 2% Nusieve-agarose gel. *a*, Ethidium bromide staining of the gel. M, DNA size marker; lane 1, PCR of DNA obtained from the first bone fragment analysed; lane 2, 'blank extraction' control; lanes 3–8, PCR of 4, 8 and 12 μl DNA from ~0.5 (lanes 3–5) and 1 mg (lanes 6–8) bone powder; lane 9, contemporary DNA control (5 μl of the PCR product). *b*, Hybridization with allele-specific oligonucleotide probes.

FS8 has been found linked to haplotype IV (one of the haplotypes associated with the higher fetal haemoglobin production) both in Turkey[15] and in Israel[14]. Moreover, the disease has been reported to run a milder course in patients with FS8/FS8 and haplotype IV, because of elevated production of fetal haemoglobin. For example, four children from Turkey (average age, 12 years) with these genotypes were reported to be rarely transfused[15].

It is therefore noteworthy that the rare codon 2 C→T polymorphism found in the Ottoman child's DNA is part of haplotype IV. This polymorphism strongly suggests that the child had elevated fetal haemoglobin. Thus the combination of homozygosity for FS8 and the codon 2 C→T polymorphism fully accounts for the severe bone pathology as well as for the unexpected survival of the child beyond infancy.

Methodology

DNA extraction. Bones were cleaned with a soft brush. The surface layer was removed by electric drill (large bit), bone powder was obtained by drilling with a sterile small burr, and DNA was isolated with Chelex 100 (Bio-Rad) solution, as described by Woodward *et al.*[11]. Two DNA extractions from ~0.5 mg and ~1 mg of bone powder were performed, in parallel to a 'blank extraction' control (containing no bone material).

Extreme care was taken to avoid contamination with contemporary human DNA. The bone samples were handled (using masks, gloves and laboratory coats) in a separate location, where no DNA analyses are performed. Ancient DNA work (extraction and setting up of the PCR reactions) was performed in two separate hoods (in different rooms), physically separated from the analyses of the PCR products and from contemporary DNA work. Disposable sterile tubes, filtered tips, and aliquots of 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 analysis of the PCR products.

PCR and sequence analyses. Four, eight and 12 μl of each DNA extract were used for PCR (a total of six tubes) along with a 'blank extraction' control and a 'no DNA' control to monitor contamination during the DNA extraction and in the PCR reaction. 'Hot-start' PCR was performed in 25-μl reactions under standard conditions (Appligene) with addition of BSA (40 ng per reaction) for 40 cycles: 1 min at 94 °C, 2 min annealing at 48 °C or 50 °C (as specified in the text) and 2 min elongation at 72 °C. Primers (9 ng each) were: 5'–GCCAATCTACTCCCAGGAGC and 3'–CCTTGATACCA-ACCTGCCCA, yielding a product of 232 bp. Aliquots (18 μl) were analysed on 2% Nusieve agarose gels. For sequence analysis, 5 μl of the PCR products and of the controls were subjected to re-PCR for additional 25 cycles. Following electrophoresis on 1% low-melting agarose gel, the bands were removed and kept frozen. Before sequencing, the agarose was melted at 68 °C and 14 μl was used for each reaction. Sequencing was performed using the PCR primers, with Sequenase Version 2 (USB).

Southern analysis. Following electrophoresis, the PCR products were blotted onto a nylon membrane (Hybond) according to the instructions of the manufacturers. Oligonucleotide hybridization was performed as described[4]. FS8 oligonucleotide was CTGAGGAGGTCTGCCGTTA and the normal was CTCCTGAGG-AGAAGTCTGC (the bases deleted in the mutant are underlined).

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1. Haldane, J. The rate of mutations of human genes. *Hereditas suppl.* **35**, 267–273 (1949).
2. Angel, J.L. Porotic hyperostosis, anemias, malarias, and marshes in the prehistoric eastern Mediterrranean. *Science* **153**, 760–763 (1966).
3. Kaplan, F., Kokotsis, G., DeBraekeleer, M., Morgan, K. & Scriver, C.R. β-thalassemia genes in French-Canadians: haplotype and mutation analysis of Portneuf chromosomes. *Am. J. hum. Genet.* **46**, 126–132 (1990).
4. Rund, D. *et al.* Evolution of a genetic disease in an ethnic isolate: β-thalassemia in the Jews of Kurdistan. *Proc. natn. Acad. Sci. U.S.A.* **88**, 310–314 (1991).
5. Oppenheim, A. *et al.* Sporadic alleles, including a novel mutation, characterize β-thalassemia in Ashkenazi Jews. *Hum. Mut.* **2**, 155–157 (1993).
6. Caffey, J. The skeletal changes in the chronic hemolytic anemias. *Am. J. Roentgenol. Radium Ther. Nucl. Med.* **37**, 293–324 (1937).
7. Stuart-Macadam, P. Anemia in past human populations. in *Diet, Demography and Disease* (eds Stuart-Macadam, P. & Kent, S.) 151–170 (de Gruyter, New York, 1992).
8. Prausnitz, M.W. Achzib. in *Encyclopedia of Archaeological Excavation in the Holy Land* (ed. Avi-Yonah, M.) Vol. 1, 9–10 (Israel Exploration Society and Massada Press, Jerusalem, 1975).
9. Smith, P., Wax, Y., Becker, A. & Einy, S. Diachronic variation in cranial thickness of Near Eastern populations. *Am. J. Phys. Anthrop.* **67**, 127–133 (1985).
10. Paabo, S., Higuchi, R.G. & Wilson, A.C. Ancient DNA and the polymerase chain reaction. *J. biol. Chem.* **264**, 9709–9712 (1989).
11. Woodward, S.R., King, M.J., Chiu, N.M., Kuchar, M.J. & Griggs, C.W. Amplification of ancient nuclear DNA from teeth and soft tissues. *PCR Meth. Appl.* **3**, 244–247 (1994).
12. Orkin, S.H. *et al.* Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. *Nature* **296**, 627–631 (1982).
13. Oner, R. *et al.* Beta-thalassemia in Turkey. *Hemoglobin* **14**, 1–13 (1990).
14. Filon, D. *et al.* Diversity of β-globin mutations in Israeli ethnic groups reflects recent historic events. *Am. J. hum. Genet.* **54**, 836–843 (1994).
15. Diaz-Chico, J.C. *et al.* Mild and severe β-thalassemia among homozygotes from Turkey: identification of the types by hybridization of amplified DNA with synthetic probes. *Blood* **71**, 248–251 (1988).