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Frequent D-loop polymorphism in mtDNA enables genotyping of 1400-year-old human remains from Merowingian graves

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Abstract. Improvements of DNA extraction and amplification techniques presently enable DNA analysis of ancient DNA (aDNA) from samples which range from several hundred years of age up to possibly 5000 years. Taking advantage of the abundance of mitochondrial DNA and its polymorphic D-loop sequence, ten individuals from multiple burial sites of the Merowingian culture (South Germany), estimated to be about 1400 years old, were genotyped to determine possible kinship. Moreover, gonosomal DNA markers from the X- and Y-chromosome were applied for sex determination of the remains. In all individuals investigated, deviations from the Anderson mtDNA consensus sequence were observed, all representing substitutions (7 transitions and 3 transversions). Although such mutations have been reported from recent populations, our study constitutes the first description of these mtDNA mutations from numerous aDNA samples recovered from multiple burial sites. The results obtained by molecular anthropology can aid in describing kinship relations and burial customs of ancient remains.

Key words: ancient DNA, kinship analysis, mtDNA sequencing.

Introduction

The human mitochondrial DNA, a circular double-stranded molecule of about 16.5 kb, has been entirely sequenced (ANDERSON et al. 1981) and codes for 37 genes. Its only non-coding region is the D-loop (1122 bp), which contains the origin of replication for the H-strand. Within the D-loop, two segments of high se-

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quence variability (HV1 and HV2) are present (LUTZ et al. 1998). The mutation rate has been estimated to be one in every 200-400 generations but new mutations have also been reported within only 18 generations (HOLLAND et al 1994b). To date now, insertions, deletions, generation of short repeats and substitutions have been observed within HV1 and HV2 (BODENTEICH et al. 1992, SAVOLAINEN et al. 1997, LUTZ et al. 1998). The polymorphic sites seem to display racial specificity (HORAI, HAYASAKA 1990).

Analysis of mtDNA – in comparison with nuclear DNA – proves highly useful in cases where the isolated DNA is severely degraded, only minute traces are available (<1 ng, BRINKMANN, WIEGAND 1997; <200 pg, SULLIVAN et al. 1992), and information about maternal inheritance is required. Its value ha been proven in testing the supposed remains of the Russian Romanov family (GILL et al. 1994).

The onset of molecular analysis of ancient DNA (aDNA) can be traced back to POINAR et al. (1982), who cloned repetitive DNA sequences from an Egyptian mummy, HIGUCHI et al. (1984) (cloning of quagga DNA) and PÄÄBO (1985) (sequencing of a 2400-year-old mtDNA). While, depending on the preservation conditions of the investigated remains, extinct species such as the mammoth were studied successfully, the authenticity of others, like miocene magnolias and 25-million-year-old bees was strongly disputed (GOLENBERG et al. 1990, CANO et al. 1992). It became obvious that only strict control criteria and highly stringent safety conditions will guarantee reliable and reproducible data on aDNA (POINAR et al. 1996).

By taking advantage of a well established isolation procedure for aDNA from skeletal remains (SCHOLZ, PUSCH 1997) and a collection of remains from two Merowingian burial sites in South Germany, we investigated kinship relations of individuals from multiple graves. For this purpose, PCR was performed using gonosomal DNA markers for sex determination and the D-loop region was sequenced for genotyping. In all 10 individuals studied, sequence deviations from the Anderson consensus sequence were noted; however, the number of individuals investigated is too small for frequency estimates of the observed polymorphisms.

Materials and methods

Bone collection

Seven bones from different individuals dating from Merowingian time were part of a collection present at the Department of Archaeo–Osteology of Landesdenkmalamt Baden-Württemberg in Konstanz and originated from graves located in Kirchheim am Ries (Germany).

Bones of the other three Merowingian individuals originated from an excavation in Niederstotzingen (Germany), and were part of a collection at the Depart-

ment of Osteology in the Institute of Anthropology and Human Genetics of the University in Tübingen.

DNA extraction

The extraction method of SCHOLZ and PUSCH (1997) with some modifications, appeared most suitable for old remains obtained from the soil of different graves with a period of burial ranging from 18 to 5000 years.

Approximately 1g of bone meal was mixed with 2.5 ml of prewarmed (60°C) mix I buffer (8.0% sucrose, 5.0% Triton X-100, 10mM EDTA, 5mM Tris-HCl (pH 8.0), 5mM ammonium acetate, 2mM magnesium acetate) and 500 µl Sephadex G-50 in a tube and vortexed vigorously for 1 min. The extraction buffer has a simple composition without proteolytic enzymes, such as proteinase K. One volume of phenol was then added to the suspension. The main macromolecular contaminants are proteins, which can be extracted with phenol. The tube was placed on a shaker at 220 rpm for 5 h at room temperature and phase separation was carried out by centrifugation at 14000 rpm for 5 min in a microfuge. The aqueous layer was transferred to a new tube, 1 volume chloroform was added, vortexed for 2 min and centrifuged as described above. The supernatant was placed into a new tube, mixed with 0.7 volume propanol and 20 µg glycogen, and carefully vortexed. Precipitation of the DNA was carried out at –20°C for 12 h. DNA was pelleted by centrifuging it at 12500 × g at 4°C for 15 min, the DNA pellet was dried at 37°C and subsequently resuspended in 1 ml mix II buffer (8% sucrose, 0.1% Triton X-100, 5mM EDTA, 1.2 M NaCl). After a second precipitation for 2 h without addition of glycogen, the DNA pellet was washed for 15 min in 70% EtOH, briefly and gently vortexed, and centrifuged once more for 10 min. The pellet was air-dried at 37°C and resuspended in 300 µl TE-buffer (10mM Tris-HCl pH 7.2-8.0, 1mM EDTA) for 12 h at 37°C. 30 µl 3M sodium acetate (pH 4,6) and 750 µl absolute EtOH were added to the 300 µl DNA solution and vortexed vigorously. Precipitation of the DNA was carried out at –20°C for 2 h. DNA was pelleted by centrifuging it at 12000 × g at 2°C for 25 min. The DNA pellet was washed with 250 µl 70% EtOH for 15-45 min. DNA was pelleted again by centrifuging at 12 000 rpm at 2°C for 10 min. The pellet was dried at 37°C for 20 min, resuspended in 10 µl TE-buffer (10mM Tris-HCl pH 7.2-8.0, 1mM EDTA) at 37°C for 12 h and stored at –20°C until further processing.

An extraction control was introduced (negative control). For this control a blank sample (lacking any template) was carried through all steps of the procedure and was used in a PCR reaction in precisely the same manner as an experimental sample.

PCR and sequencing

PCRs of STRs (Amelogenin, DYS390) were carried out in a volume of 25 µl with 10 µl of DNA extract (100% of extract), with 10X-buffer, with primers and

dNTPs from the Kits of SERAC (Manfred R. Hoffman. Serologische Reagenzien GmbH). The amounts of BSA (160-200 μg/ml), Taq-polymerase (1-1.75 U) were individual for each locus indeed for different samples. We cannot generalize STR-PCR-cycling conditions for all samples examined. There were in each STR-system samples with individual demands. As a rule, the Booster-PCR with a total of 35-45 cycles was the most efficient.

However, there were regular (for all our samples) PCR conditions for the mtDNA-fragments. The PCR reaction mix (in a reaction volume of 25 μl) contains in all cases 10X-buffer with 2.0 mM $MgCl_2$ (pH 8.6), 0.2mM dNTPs, 0.5 μM of each primer, BSA (200 μl/ml) and 1U Taq-polymerase.

PCR amplification of mtDNA control region (D-loop) was performed using four primer sets:
(1) L 15990 and H 16263, amplified a fragment of 273 bp;
(2) L 16267 and H 16498, amplified a fragment of 231 bp;
(3) L 00029 and H 00246, amplified a fragment of 217 bp;
(4) L 00340 and H 00617, amplified a fragment of 277 bp.

Cycling conditions for the 273 bp and 231 bp fragments:
1 cycle: 94°C/1 min, 35 cycles: 94°C/45s, 54°C/1min, 72°C/1min, 1 cycle: 72°C/10min.

Cycling conditions for the 217 bp fragment:
1 cycle: 94°C/1 min, 35 cycles: 94°C/45s, 55°C/1min, 72°C/1min, 1 cycle: 72°C/10min.

Cycling conditions for the 277 bp fragment:
1 cycle: 94°C/1 min, 35 cycles: 94°C/45s, 51°C/1min, 72°C/1min, 1 cycle: 72°C/10min.

The amplified fragments of mtDNA were purified using Microcon-100 ultrafiltration.

We used for cycle sequencing an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS from Perkin Elmer Applied Biosystems.

Electrophoresis and sequence analysis were performed with an ABI Prism 377 DNA Sequencer (PE Applied Biosystems).

Both strands of the amplified regions were sequenced and compared with each other to ensure fidelity of the data. Internal controls, positive and negative controls were also incorporated in the analysis. The ANDERSON sequence (1981) was used as the reference sequence.

For complete DNA analysis of one individual at least 13 g of bone powder (amounting to about 13 extracts) are required.

Controls

Due to a variety of possible contaminants and artificial PCR products, a wide variety of controls was introduced. Species-specificity was verified by adding defined human DNA (cell line K562 and the experimenters) and bovine DNA. Mock ex-

tracts and complete reactions minus DNA were routinely included. The extracts were repeated from the same bone samples. Only complete sequences (H- and L-strand) were taken for the D-loop polymorphism. Classical precautions like UV surface decontamination and local separation of the extraction and PCR steps were self-evident.

Results

To monitor the reliability of the DNA extraction step, recent and historical bones (from 300 to 5000 years old) were used for DNA isolation and subsequent PCR to determine the polymorphism of chromosomal loci. Usually biphasic PCR proved necessary to obtain visible bands in gel electrophoresis. The gonosomal markers amelogenin and DYS390 yielded bands that allowed sex determination of the samples investigated (Table 1). However, we collected inferior signals with DYS19 and DYS393, so, studies with these markers were discontinued (data not shown). Among the 10 individuals, 5 were males and 5 females.

Table 1. Sex determination of skeletal remains

Origin	Person	Morphology	DNA	Amelogenin	DYS390
Kirchheim am Ries	81	male	male	male	22
	87	male	male	male	22
	88	female	female	female	–
	279	female	female	female	–
	282	?(child)	female	female	–
	286	?(child)	male	male	22
	289	male	female	female	–
Niederstotzingen	3a	male	male	male	18
	3b	male	male	male	24
	3c	male	female	male	–

To avoid sequence discrepancies from the published Anderson consensus, which may be due to sequencing artifacts like compression zones, the aDNA sequences were always composed by reading both strands. The alterations in all 10 individuals are listed in Table 2. Identical polymorphisms, e.g. in individuals 81, 87, 88 (from one grave) and 282 and 289 with deviation to 279 and 286 (all four of them from the second grave), allow a grouping into kinships. It was found that child 286 was related to the adult female 279 (mother?) and not to the rest of the female individuals.

In the Niederstotzingen grave, mtDNA sequence comparison and Y-marker results proved that both males (3a, 3b) are not related, whilst 3b and 3c share

Table 2. Deviations in the D-loop sequence of mtDNA in all tested individuals. A letter indicates a substitution relative to the reference sequence of ANDERSON (1981)

Origin	Person	Position 16356	Position 16358	Position 72	Position 73	Position 195	Position 499	Position 527
Kirchheim am Ries	81	C	–	–	G	C	A	–
	87	C	–	–	G	C	A	–
	88	C	–	–	G	C	A	–
	279	–	G	C	–	–	–	–
	282	C	–	–	G	C	A	–
	286	–	G	C	–	–	–	–
	289	C	–	–	G	C	A	–
Niederstotzingen	3a	–	–	C	–	–	A	G
	3b	C	–	C	–	–	A	G
	3c	C	–	C	–	–	A	G

the same mtDNA polymorphic sites. Sex determination surprisingly revealed 3c to be a female, while the historical interpretation was that of a young warrior since all three individuals were burried with swords.

Discussion

DNA sequence polymorphisms, like base substitutions, can be detected by hybridization with specific probes or by direct sequencing. WALLACE et al. (1991) demonstrated that the latter detects four times as many polymorphic sites as RFLP analysis. While hybridization with sequence-specific oligonucleotides will differentiate between individual DNAs, it is less sensitive when only minute amounts of DNA are available; direct sequencing of the D-loop region remains the method of choice (HOLLAND et al. 1994a). Its powerful sensitivity, however, renders this technology susceptible to contaminants, like foreign DNA.

Due to mtDNA sequence similarities in a variety of species, both eukaryotic and prokaryotic, it was mandatory to prove the *Homo sapiens* specificity of the isolated material. A 120 bp fragment from region V was PCR-amplified as a strictly human-specific sequence (CATTANEO et al. 1999). Further, to exclude contamination by individuals involved in the testing process, their mtDNA was sequenced parallel to the aDNA samples. As a standard procedure, possible surface contamination of the bone fragments was removed by routine steps like UV-irradiation and surface abrasion.

All PCR steps contained positive controls to test primers and enzymatic conditions, and corresponding negative controls. A possible drop-out of the Y-chromosomal allele of the amelogenin gene can be discounted since it occurs world-wide at a frequency of 0.6% (SANTOS et al. 1998). The fact that among the 5 male samples, 3 allelic forms for DYS390 (18, 22 and 24) were present, proves the individuality of the mtDNAs and argues against a general contamination. Similarly, this fact holds true for the D-loop polymorphisms since 4 sequence variants were found in the group of the 10 studied individuals and none of them displayed the Anderson consensus sequence.

The data provide two interesting facts. First of all, in addition to the remarkable efficiency of the molecular assays of 1400-year-old aDNA, the mtDNA sequence proved to contain sequences deviating from the Anderson consensus and thus allowing a grouping of individuals into likely kindreds. Extension of such studies, possibly including highly polymorphic STR markers, may even allow a precise genealogy of such findinas. Secondly, sex determination, sometimes not feasible by morphological criteria, was successful in all cases. In one particular instance, a feminine sex was proven, despite the fact that, based on cultural evidence, a male individual was expected. Such molecular data will surely aid in improved understanding of human remains, supporting various anthropological disciplines. Using modern human genetic tools we can envision a wide-spread flourishing of molecular anthropology.

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