DNA Analysis on Biological Remains from Archaeological Findings - Sex Identification and Kinship Analysis on Skeletons from Mitterkirchen, Upper Austria

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Zusammenfassung

Der Nachweis und die Charakterisierung organischer Komponenten in historischen und archäologischen Funden, im Speziellen der DNA (Desoxyribonucleinsäure), hat sich neben anderen naturwissenschaftlichen Analysemethoden (Radiocarbon-Datierung, Dendrochronologie, Radiologie, Isotopenanalysen, etc.) als ein wichtiges Teilgebiet der archäometrischen Analytik etabliert.

Der Zugriff auf die genetische Information in gealterten biogenen Überresten erlaubt prinzipiell Aussagen über alle Eigenschaften, die sich in irgendeiner Form von DNA / Erbsubstanz manifestiert haben (biologisches Geschlecht, Verwandtschaftsbeziehungen, Erb- und Infektionskrankheiten, etc.).

Im Folgenden wird eine allgemeine Übersicht über die Analyse alter DNA und eine kurze Anleitung zur Probennahme und Aufbewahrung gegeben. Weiters werden mögliche Anwendungen diskutiert und eine Fallstudie über die vorläufigen Ergebnisse der molekularbiologischen Untersuchung an Skelettüberresten aus Mitterkirchen, Oberösterreich, präsentiert.

Abstract

*The detection and characterisation of organic compounds, especially DNA (**D**eoxyribo**n**ucleic **A**cid), in historical and archaeological remains has become an important field among other scientific techniques applied to ancient findings (such as radiocarbon dating, dendrochronology or isotope analysis).*

Reading the DNA sequence of genetic material preserved in aged biological matter allows us to gain information on anything that has manifested itself by means of DNA (sex, species, kinship, diseases, etc.)

We present a general overview on the technical procedure of ancient DNA analysis, a short guide to sample collection and storage, possible applications and a case report referring to the results of a preliminary study on skeletal remains from Mitterkirchen, Upper Austria.

Introduction

Until the 1980´s most researchers were convinced that no detectable genetic information is preserved in ancient remains due to the rapid decay of bio-organic molecules (Eglinton *et al.* 1991; Lindahl 1993). First reports on the characterisation of ancient DNA (Higuchi *et al.* 1984; Higuchi *et al.* 1987; Paabo 1985; Paabo 1986) were very controversial, apart from the fact that the laboratory work required a high level of technical and financial resources.

The invention of the PCR technique (**P**olymerase **C**hain **R**eaction) in the mid-1980´s (Saiki *et al.* 1988) made possible the (artificial) amplification of one or

more certain units of genetic material to a detectable amount to enable access to the information encoded in the DNA molecule (Krawczak 1994). Due to the exponential nature of DNA amplification during the PCR process, minute amounts down to only *one* intact unit (molecule) of the required genetic information may be enough to read a DNA sequence or structure (Herrmann *et al.* 1994; Hummel 2003). Hence, molecular archaeology does not mainly focus on the accurate determination of a certain compound of interest (i.e. as seen in radiocarbon dating), but on the information encoded by the DNA molecule.

As mentioned above, DNA characterisation (theoretically) allows reading any information referring to genetic features such as biological sex, kinship (family pedigree, intrapopulation, interpopulation, evolutionary context,...), species as well as hereditary and infectious diseases (Hummel 2003). Aside from these applications (long time deceased), individuals can be identified by DNA typing at a very high level of accuracy (Anslinger *et al.* 2001; Egeland *et al.* 2000; Gill *et al.* 1994; Ovchinnikov *et al.* 2000). Therefore ancient DNA typing is not only useful to validate preliminary findings (anthropology, archaeology, palaeontology, palaeopathology, migration data,...), but also to determine completely new sets of data such as the biological sex in preadolescent individuals and children (i.e. (Faerman *et al.* 1995)) as well as in cases of small fragmented findings (i.e. (Schultes *et al.* 1997)), family and population kinship (i.e. (Gerstenberger *et al.* 2002; Gerstenberger *et al.* 1999; Hummel 1997; Hummel *et al.* 1996)), evolutionary background (i.e. (Gibbons 1993; Watson *et al.* 1997)) and diseases (i.e. (Haas *et al.* 2000; Haas *et al.* 2000; Nerlich *et al.* 2002; Pusch *et al.* 2004)) with minor or no morphological consequences...

Sample collection and storage

(Cann *et al.* 1993), (Hummel 2003)

One of the major pitfalls in the field of ancient DNA analysis is the high risk of contamination by introduction of foreign, non authentic genetic material to the objects of investigation. This may occur even when only touching the sample with uncovered skin – body fluids (blood, saliva, etc.) are highly contaminous, respectively.

In respect to ancient DNA analysis, excavators should wear a whole protection including a face mask and rubber gloves – since this is not feasible in every day archaeological field work we recommend at least the utilization of rubber gloves when the remains are collected from the excavation site. In many cases, the samples have been unprotectedly touched by several individuals during the excavation and subsequent investigation and treatment, which requires extensive precautions during sample preparation and DNA isolation in the laboratory (see below).

Samples must not be washed or sealed in airtight receptacles (i.e. zip lock bags) to avoid further contamination, washing out of genetic material and – most important – to avoid microbiological growth downstream to the sample collection (bacterial and fungal activity destroys the physical structure of the remains (tunneling) as well as the molecular integrity of the DNA molecules).

Single samples for ancient DNA analysis are recommended to be collected in paper bags or envelopes, labelled, locked with staples and stored at a dry, remote place until opened in a suitable, non contaminous laboratory environment.

If available, in general a total of ten samples should be taken from each individual (two sets of corresponding sets, i.e. 2 skull fragments, 2 teeth, 2 small bones from the hands, from the feet,...) since not all samples yield analyzable DNA. The size of the sample should be at least around one gram to yield enough material for a complete analysis. Major fragments up to whole bones are good as well – in this case only a small portion (0,2 to approx. 1 gram) of tissue is removed from the sample.

Forensic DNA typing applied to archaeological remains allows access not only to different levels of kinship but also to ensure the authenticity of the data found. Therefore all affected personnel (excavators, anthropologists, lab staff,...) should provide an (anonymous) reference sample (Hummel 2003) to exclude modern contamination by experimenters (see below).

Sample Pretreatment, Physical and Chemical Breakup

Once in the laboratory, the samples must be stored and treated under sterile, non contaminous conditions: Any surface (instruments, containers, handling devices,...) have to be free of any DNA or DNA degrading agents (DNAses, anorganic substances,...). Any procedure has

to ensure that no contamination or further DNA decay can occur (Hummel 2003)!

As mentioned above, ancient DNA samples are most likely contaminated, especially when they were not taken under „ancient DNA conditions". Naturally, most contaminations occur on the samples´ surface, many samples still carry remains of soil – therefore the outer layer has to be removed (at least at the area of sample preparation): Usually this is done with a rotating wire brush, a sand blast device or similar procedures (Niederhauser *et al.* 1994;Van Helden P.D. *et al.* 1992); (Hummel 2003).

Since bones and teeth constitute the main volume of available tissue, a physical break-up has to be performed prior to DNA-extraction: most procedures recommend a gross break-up in a mortar following pulverization with a swing mill. With larger objects bone powder can be collected directly from the (locally pretreated) sample with a drill. Remaining material can be restored physically if required (i.e. for exhibitions).

For the following analysis, the DNA has to be purified and transformed into an aqueous solution (Kiesslich *et al.* 2002): about 0,2 to 1 gram of bone powder is taken up in an aqueous solution of EDTA (Ethylene diamine tetra acetate) to perform decalcification (chemical breakup of bone or tooth matter) followed by enzymatic treatment with ProteinaseK (breaks up protein compounds associated with DNA) and further purification to remove „non DNA" compounds such as salt, protein fragments or soil components – all potentially likely to inhibit (Goodyear *et al.* 1994; Hall L.M. *et al.* 1992; Herrmann, Hummel 1994; Montiel *et al.* 1997) the subsequent amplification (multiplication) of genetic areas of interest in the (enzymatically mediated) Polymerase Chain Reaction procedure.

DNA amplification, detection and characterization (Lottspeich *et al.* 1998; Newton *et al.* 1994)
As indicated above, the PCR process appears to be the key technique in the field of molecular archaeology. Depending on storage conditions and time, only minute amounts of intact genetic information (a few 100 up to 10000 intact molecules) can be expected in aged samples (Collins *et al.* 1999; Herrmann *et al.* 1982;Vass *et al.* 2002;Verhoff *et al.* 2004) – a level far below the detection threshold of conventional molecular techniques like (capillary)electrophoresis (Butler 2001). The PCR process basically refers to an exponential, selective and exclusive amplification of areas of interest on a DNA molecule. Within a cyclic procedure of heating and cooling, the number of molecules are doubled after each cycle – the performance of a limited number of cycles (30-50, (Rameckers *et al.* 1997)) yields millions of identical copies carrying the desired information suitable to be read with electrophoretic methods.

(Capillary)electrophoresis (Butler 2001) allows the exact measurement of fragment length (i.e. for kinship analysis) as well as direct sequencing of amplified DNA molecules (i.e. for species identification, palaeopathology or population dynamic studies).

Kinship analysis, DNA information structure and interpretation (Brown *et al.* 1994; Gerstenberger, Hummel *et al.* 2002; Scholz *et al.* 2001; Schultes *et al.* 2000)
DNA based kinship analysis (Butler 2001; Krawczak 1994) within a family group (as seen with regular paternity tests) refers to certain structures of genetic information, known as STRs (**S**hort **T**andem **R**epeats). These regions or loci within the genome have no known physiological features – they consist of small „words" by means of DNA, tandemly arranged in a varying number of repeats. However, the number of repeats is specific for each individual and inherited according to Mendel´s laws. Therefore, one individual carries exactly 50% maternal and paternal genetic markers, here STRs; in other words: one´s individual constitution refers equally to the mother´s and the father´s genotype – in case one individual shares at least 50% of genetic markers with another one, there is a high probability of paternity between them (see below).

Contamination control and data authentification (Hummel *et al.* 2000; Loy 1997; Niederhauser, Hofelein *et al.* 1994; Schmidt *et al.* 1995; Spencer *et al.* 2004; Yang *et al.* 2003) remain to be controversial within molecular archaeology, especially since, in case of human ancient DNA, the experimenters represent the major source of contaminations. Therefore, extensive measures to avoid foreign DNA introduction have to be carried out: beside a clean, DNA free working environment in the laboratory, a certain set of control extractions has to be carried out to demonstrate the

absence of non authentic DNA during the whole procedure in any applied reagent.

Additional DNA typing of all persons that may have transferred biological material to the sample (see above) is highly recommended – if not mandatory.

Hence, ancient DNA data is (likely) to be authentic if:

1. no contaminating DNA was detected during the whole procedure,
2. no „ancient" data matches any of the experimenters
3. and (optional) an „ancient" kinship was found.

What can ancient DNA tell us (Brown, 1998)? – some major applications (Hummel 2003)

Sex identification (Brown 1998; Götherström 2002; Hummel *et al.* 2000; Lassen *et al.* 2000; Nakahori *et al.* 1991; Schmidt *et al.* 2003; Vernesi *et al.* 1999): especially when the individual of interest is subadult/infantile or the remains are incomplete (i.e. no coxa, no or destroyed skull) morphometric sex identification remains doubtful or is not possible. In some cases, even burnt or charred remains are feasible for DNA analysis. The detection of different structures on the X and the Y chromosome (Faerman, Filon *et al.* 1995; Stone *et al.* 1996) respectively offers a reliable method to identify the individuals´ biological sex.

Kinship analysis (Gerstenberger, Hummel *et al.* 2002; Gill, Ivanov *et al.* 1994; Herrmann, Hummel 1994; Hummel 1997; Hummel *et al.* 1997; Hummel *et al.* 1999): Accessing the kinship between two or more individuals by means of DNA introduces a new feature into the scientific analysis of ancient remains. The application of paternity testing methods may enable the reconstruction of a complete pedigree within an excavation site. Y-chromosomal DNA analysis (only with males) allows the definition of paternal genetic lineages and, moreover, a gross geographic correlation of the found genotype (referred as „ethnic estimation", i.e. Scandinavian, southern European,...) as well as information on population and migration structure dynamics.

Species identification (Amendt *et al.* 2004; Bellis *et al.* 2003; Hsieh *et al.* 2001; Parson *et al.* 2000): in cases of undefined species as well as in connection with fields like palaeoecology, pollen analysis and other areas that require exact species identification, DNA analysis on species specific sequences within the individuals´ genetic material provides an additional, independent way to access respective data.

Palaeopathology (Hummel *et al.* 1995; Jankauskas 2002; Nerlich *et al.* 1997; Padberg 1992; Persing *et al.* 1990; Rafi *et al.* 1994; Taylor *et al.* 1997; Zhu *et al.* 1998): Since infectious and hereditary diseases represent a major issue in (human) life, the detection of pathogens (viruses, bacteria,...) provides valuable information on everyday life and individual constitution of a person or society as well as for current epidemic research (malaria, plague, tuberculosis, syphillis, anemia, hemochromatosis,...).

case report:

Paternity Testing on the Remains from Mitterkirchen, Upper Austria

General remarks on the finding

The excavations of the early iron age burial site at Mitterkirchen (Upper Austria) were performed in 1981–1990 by Manfred Pertlwieser (head of the Prehistoric department, OÖ. Landesmuseum) and his collegue Vlasta Tovornik together with museum employees and numerous locals from the surrounding farms. The findings were transferred to the depot of the OÖ. Landesmuseum in Linz/Wegscheid and stored in cardboard boxes at room temperature. Human remains were not further treated or cleaned. Tooth samples from the individuals I/3, II/1 and X/1 were taken for DNA based sex identification (Götherström 1998).

For the present study, we focused on 5 individuals (see below) of special archaeological interest: The oval barrow HÜ-X contained two tombs: a double burial (individuals HÜ-X/2-A and HÜ-X/2-B) and a wagon grave of a female (HÜ-X/1), both richly equipped with burial objects. Next to the tomb of HÜ-X/1 a crouched inhumation burial (HÜ-X/H = HÜ-X/3) was found without any burial objects. Apart from barrow X, samples were taken from the primary central tomb of barrow I (HÜ-I/8, richly equipped as well).

DNA Analysis

Samples were stored, cleaned and pre-treated according to generally accepted guidelines for ancient DNA analysis.

Ancient DNA was isolated utilizing decalcification and subsequent filter dialysis, followed by PCR and capillary electrophoresis (for details see (Kiesslich, Radacher et al. 2002)).

Results

Samples from individual HÜ-X/2-A, individual HÜ-X/2-B and individual HÜ-X/2H (HÜ-X/3) did not yield any detectable DNA.

Two teeth from the individual HÜ-X/1 showed a partial DNA profile (incomplete genotype).

Individual HÜ-I/8 yielded a partial DNA profile for a molar, part of a patella as well as from a small bone fragment. Additionally, a complete DNA profile was obtained from another small bone fragment.

The individual HÜ-X/1 proved to have a female (corresponding a previous analysis, (Götherström 1998)), the individual HÜ-I/8 a male genotype – both confirmed by anthropological and archaeological findings.

Since two paternally related individuals show at least 50 % corresponding DNA markers, locuswise comparison of genetic data from HÜ-X/1 and HÜ-I/8 indicates a father / daughter or mother / son relationship between these two individuals (see table below).

Biostatistical analysis of given data revealed 99.2232% probability for a parental relationship, 0.7287% for siblings and 0.0481% for no relation.

There was no match with any potentially contaminating persons.

Discussion

The genotype for individual HÜ-I/8 (male) is regarded as authentic (no contaminations during the procedure, corresponding data from 3 samples originating from the same individual, multiple reproduction of data).

Aside from the absence of contaminations, present data for individual HÜ-X/1 (female) is still incomplete – the analysis of another set of samples should be done to confirm and validate the data shown in the table below – however this data appears to be authentic since a kinship correlation was found with individual HÜ-I/8.

Outlook

For the male (individual HÜ-I/8), additional analysis of the Y-chromosome (Bradman *et al.* 2002; Kayser *et al.* 2000; Passarino *et al.* 1998; Redd *et al.* 2002) is currently being done for the assessment of a paternal lineage and ethnic estimation (http://yhrd.org) (see above).

DNA from both individuals is currently being analysed to see whether or not these individuals carry genetic markers responsible for hemochromatosis (Datz 2000; Datz *et al.* 1997; Kazemi-Shirazi *et al.* 1999) (a common contemporary hereditary disease in populations derived from the ancient celts); we also suggest to test respective DNA for infectious diseases by PCRs usually applied in clinical diagnosis (Herrmann, Hummel 1994; Hummel, Herrmann 1995; Jankauskas 2002; Nerlich, Haas *et al.* 1997; Persing, Telford *et al.* 1990; Rafi, Spigelman *et al.* 1994).

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autosomale Loci	D8S1179		D21S11		D7S820		CSF1PO	
Ind. HÜ-X - SK 1 *(MK-I)*	*12*	*15*	*27*	*32,2*	*8*	*10*	*0*	*0*
Ind. HÜ-I - SK 8 **(MK-I)**	**10**	**12**	**31**	**32,2**	**8**	**9**	**11**	**11**
	D3S1358		TH01		D13S317		D16S539	
Ind. HÜ-X - SK 1 *(MK-I)*	*16*	*17*	*6/9/9,3*		*11*	*13*	*10*	*12*
Ind. HÜ-I - SK 8 **(MK-I)**	**15**	**16**	**6**	**9**	**11**	**14**	**12**	**12**
	D2S1338		D19S433		vWA		TPOX	
Ind. HÜ-X - SK 1 *(MK-I)*	*0*	*0*	*14,2*	*15*	*14*	*19*	*0*	*0*
Ind. HÜ-I - SK 8 **(MK-I)**	**23**	**24**	**14**	**14,2**	**14**	**15**	**8**	**9**
	D18S51		AMEL		D5S818		FGA	
Ind. HÜ-X - SK 1 *(MK-I)*	*14*	*15*	*X*	*X*	*11*	*12*	*21*	*23*
Ind. HÜ-I - SK 8 **(MK-I)**	**(13)**	**(13)**	**X**	**Y**	**11**	**11**	**21**	**21**

table: locuswise comparison of genotypes found; source: Kiesslich, Jan, 2002

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