ANTİK DNA VE ADLİ TIP KARŞILIKLI FAYDALARI: SANAL BİR ANTİK DNA ÇALIŞMASI İLE İLGİLİ PRATİK ÖRNEKLEME VE LABORATUVAR KILAVUZU

Ancient DNA and Forensics Mutual Benefits: A Practical Sampling and Laboratory Guide Through a Virtual Ancient DNA Study

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ABSTRACT

Genetic information discovered, characterized for and used in forensic case-works and anthropology has shown to be also highly useful and relevant in investigating human remains from archaeological findings. By technical means, forensic and aDNA (ancient Deoxyribonucleic acid) analyses are well suited to be done using the same laboratory infrastructures and scientific expertise referring to sampling, sample protection, sample processing, contamination control as well as requiring analogous technical know how and knowledge on reading and interpreting DNA encoded information. Forensic genetics has significantly profited from aDNA-related developments (and vice versa, of course!), especially, when it comes to the identification of unknown human remains referring to the detection limit. Additionally the tremendous developments of analyzing chemistry and kits as well as instruments in forensics opened the whole panel of reading human and nonhuman DNA for historians and archaeologists but also for anthropologists. Ancient DNA / molecular archaeology, however, is not limited to the comparatively restrictive set of information as usually employed in forensic case work analyses but can also be applied to phenotypical markers, ethno-related genotypes or pathological features.

In this review the authors give a general overview on the field of ancient DNA analysis focussing of the potentials and limits, fields of application, requirements for samples, laboratory setup, reaction design and equipment as well as a brief outlook on current developments, future perspectives and potential cross links with associated scientific disciplines.

Key words: Human DNA, Ancient DNA, Forensic DNA typing, Molecular archaeology, Application.

ÖZET

Adli olgu çalışmalarında ve antropoloji alanında kullanılmakta olan, keşfedilen genetik bilgilerin arkeolojik kalıntılardan elde edilen insan kalıntılarının incelenmesiyle ilişkili ve son derece faydalı olduğu gösterilmiştir. Adli DNA ve aDNA(antik DNA) analizleri teknik anlamda numunenin bilimsel uzmana sunulması, numunenin korunması, numunenin işlenmesi, kontaminasyonun kontrolü ile birlikte uzmanlık teknik ve bilgisi gerektiren, kodlanmış DNA'nın okunması ve yorumlanması gibi işlemler için bazı laboratuar alt yapılarının kullanılmasıyla uygun hale getirilir. Adli genetik, özellikle tespit edilebilir sınırlarda bilinmeyen insan kalıntılarının kimliklendirilmesi söz konusu olduğunda aDNA ile ilişkili gelişmelerden önemli derecede faydalanmıştır (tabi ki karşılıklı olarak!). Tarihçiler, arkeologlar ve aynı zamanda antropologlar için kitler ve kimyasal analizlerdeki muazzam gelişmeler ile birlikte Adli Tıp araçları insan ve insane ait olmayan

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tüm DNA panellerinin tiplendirilmesini başlattı. Antik DNA/moleküler arkeoloji sadece adli olgu çalışma analizleri gibi bilgi dizileri ile sınırlı değildir, aynı zamanda fenotip markerları, etnik ilişkili genotipler veya patolojik özellikler için de ayrıca uygulanabilir.

Bu derlemede yazarlar antik DNA analizinin potansiyelini ve sınırlarını, uygulama alanlarını, numuneler için yapılması gerekenleri, laboratuar kurulumunu, ekipman dizaynı ile birlikte güncel gelişmeler üzerine kısa bir bakış, gelecek perspektifleri ve ilişkili bilimsel disiplinlerin potansiyel çapraz bağlantıları ile ilgili de genel bir bakış açısı vermeye çalışmıştır.

Anahtar kelimeler: İnsan DNA'sı, antik DNA, adli tıbbi DNA tiplemesi, moleküler arkeoloji, uygulama.

INTRODUCTION

Deoxyribonucleic acid represents a unique target molecule in trace analysis in the course of forensic but also historically or archeologically relevant samples. Conventional methods such as radiocarbon dating, organic and inorganic analyses aim for a certain isotope, molecule or group of molecules and their derivates, determining a precise amount or concentration in the samples under investigation down to its detection limit. Certainly, DNA analysis requires a minute amount of molecules - however not the concentration is the decisive parameter but the information encoded. Theoretically, a single intact target molecule may be sufficient for successful genotyping. Ancient DNA (aDNA) analysis has emerged since the mid 1980s employing PCR (polymerase chain reaction) -based DNA analysis of minute amounts of DNA (1) preserved in historically / archaeologically relevant samples (i.e. (2)). Since then, aDNA-analysis has shown to be a powerful tool in confirming and amending historical information from prehistoric times up to recent history. Due to the nature of information encoded in the DNA molecule it allows the assessment of individual and group features, such as the biological sex, family and population kinship, ehtno-geographical estimation and provenancing as well as hereditary and some infectious diseases (3). By technical means, aDNA or molecular archaeology respectively, is closely related to forensic DNA typing, usually utilizing the same or similar techniques and requiring analogous laboratory infrastructure; hence, these fields have significantly benefited from mutual exchange. As seen from this point of view, ancient DNA analysis / molecular archaeology has to be clearly distinguished from palaeogenetics: Referring to the hypotheses under investigation, aDNA research is to be seen as a historical discipline, utilizing an alternative method of reading (a certain kind) ancient information (4). Seen from the same point of view, palaeogenetics is clearly suited to the field of evolutionary biology and associated fields - nevertheless, both disciplines share commons such as lab requirements and sometimes even the samples.

As mentioned above, the invention of the Polymerase Chain Reaction (PCR) by Kerry Mullis and colleagues in the mid 1980s represents the crucial event for DNA trace analysis: This technique allows one to artificially multiply minute amounts of DNA to a reasonable amount suitable for DNA-sequencing and fragment length analysis for the assessment of the respective genetic information. Limits of PCR are seen at a high degree of DNA degradation or fragmentation as well as in chemical alterations (i.e. (5), (6) or (7)), both due to digenetic and taphonomic effects leading to a null-result. Naturally, hard tissue remains represent the most frequent sample / tissue type under investigation. Additionally, bones and teeth represent an excellent substrate for the protection of genetic information due to its microanatomy(8).

Generally speaking, any information manifested in DNA and preserved throughout history can be - theoretically - assessed. Unfortunately there is no feature on or associated with the DNA molecule correlating with the PMI (post mortal interval) so it can't serve as a tool for dating. Certainly, DNA integrity declines time-dependently but there are other factors such as water content, temperature, presence/absence of oxygen, variability of the microclimate, etc. affecting the DNA's molecular integrity additionally to the PMI i.e. ((5), (9) and (11)) and hence tampering with the temporal correlation. Several studies set out to evaluate a independent parameter correlating with the DNA quality / readability, such as nitrogen content, physical preservation or amino acid racemisation (i.e. (10) or (11)). Since these did not result in a reliable 'pre-test' system and require an additional analytical methodology, the authors suggest focussing on 'promising' samples for a first try to evaluate the molecular archaeological potentials of a certain finding.

The following chapters provide a guide through a virtual molecular-archaeological case involving human remains from the first information on findings of interest to the final assessment and interpretation of results. There will be some recommendations especially involving sampling and DNA extraction - the authors want to state,

that these chapters are based on their own 15 years of experience working on DNA from ancient and recent human remains. However, some readers may not fully agree with our recommendations and/or statements due to their own experience and expertise (12). Despite of archaeology-specific issues, statements and recommendations the following chapter are to be taken into account also in forensic casework involving (unknown) human remains: assigning unknown deceased in case of post mortal alterations such as long PMI (post mortal interval), fire, explosions, exposure to nature or animal scavenging as well as cases of exhumation or working on mass graves from recent and - of course historical conflicts. Mass disasters with a great number of fatalities underlie special regulations (DVI / disaster victim identification guidelines, i.e.(13).

PREANALYTICS

There are some preanalytical measures recommended for a valid ancient DNA analysis on recent and historical or archaeological osseous remains: The accounted archaeologist / excavator should find a partner from a forensic or ancient DNA laboratory. The respective person should be involved from the first day of planning the excavation including funding and logistic support for the upcoming analyses, a written agreement is recommended.

Briefing and Referencing

Before the first dig is done, the whole excavation team should be briefed on the basic principles of aDNA and other scientific analysis focussing on what has to be done as soon as osseous remains are unearthed. There should be a list of persons given to the DNA expert in order to prepare sampling kits for the collection buccal swab samples from all affiliated persons in order to produce a set of reference DNA profiles to be compared to the prospective DNA results from the archaeological findings expected. Certainly, this can be done anonymously - the key issue is to ensure the identification of modern DNA contamination. The authors recommend preparing a paper with brief information on the purpose of the reference sample including a written declaration of confidentiality on the samples and the outcomes of the analysis. Individual genetic features underlie the donors' privacy of information - this has to be respected, even in case a person involved refuses to give a reference sample due to individual concerns. However, a missing reference sample can significantly handicap DNA data validation.

Sample Protection Measures

According to the authors' experience the risk of contaminations by excavators is not too high but can be reduced significantly if the excavators use mouth protection and disposable gloves as soon as the first bone fragment is discovered. These measures should be maintained until the whole skeleton(s) is recovered and sealed in bags and / or boxes. Some researchers recommend even the use of full body protection cover-alls during the recovery - this, of course is advantageous in the course of contamination prevention but most likely not workable due to the climatic and weather conditions during archaeological excavations. In any case, the excavator / scientist handling the remains represents the utmost danger to the samples due to contamination and/or mishandling.

Sampling and Sample Storage, On-Site Measures

Some studies have shown, that immediate recovery, on-site-sampling and deep freezing (less than approx. -40°C) of bone and tooth samples are the best way of sample storage to prevent DNA degradation until the DNA extraction process is started (14). (Unfortunately a deep freezer is usually not part of the excavation equipment.) Hence, we recommend using a cool box as usually used for beverages or a similar storage device to collect the samples. In this case the samples should be stored in airtight plastic bag (i.e. zip-lock bags) but only if unbroken refrigeration or cooling can be provided until the samples arrive in a dedicated storage facility or DNA laboratory.

If no such measures as described above are available or appear to be practical, we recommend using paper bags or envelops to collect bone and tooth samples, completed sample bags should be stored in cardboard boxes; this is especially recommended for moist samples, since the presence of water is a prerequisite for enzymatic activity a microbial growth, both factors accelerating DNA decay. Storage in paper/cardboard enables the humidity to evaporate and reduces the danger of DNA damage until the samples undergo further analyses. By technical means, samples from the same individual / skeleton can be collected in a single bag. The amount of samples available of course depends on the general condition and completeness of the finding. Given, an almost complete skeleton is found, take 2 sets of 5-7 different samples scattered over the whole skeleton. Focus on peripheral bones from hands and feet first but also from areas with a comparatively low amount of soft tissue (in premortal condition) or thick solid compacta (i.e. femur or humerus

shafts) as well as teeth. Be aware of commingling, especially with minor body parts, in case of more than one individual is found in an internment. Due to the authors experience, samples from the peripheral bones are more likely to yield good quality DNA than proximal bones; we assume, that the presence of larger amounts of soft tissue is prone to go along with a more vigorous decomposition - hence the DNA-damaging processes are more intense and occur for a longer time than in areas with low amounts of soft tissue such as fingers. Generally, we recommend involving the DNA expert in the excavation at least for the recovery of the skeletal remains. According to the authors experience the best way is a collaborative effort of the DNA specialist together with the anthropologist to avoid 'sampling conflicts'; moreover sampling for additional analyses such as radiocarbon dating or isotope analysis should be considered throughout the sampling and recovery efforts. Restricting the number of persons involved during the unearthing reduces the risk of multiple contaminations. According to the procedures during police crime scene investigation some of the archaeologists can be trained for the sampling process alternatively representing the 'on-site-DNA-task-force'.

Referring to the most sensitive analytical target - DNA - the samples should be stored cool, dry and under a constant microclimate; significant and repeated changes in temperature (i.e. freeze-and-thaw cycles) are to be avoided. In any case, the samples are not immediately (the same day) transferred to a dedicated laboratory, airtight storage compartments should be avoided; as indicated above, paper bags and cardboard boxes provide sufficient gas exchange to relief the moist and protection of the samples from modern DNA contamination. In contradiction to a usual and long time praxis in archaeology as well as in recovery of osseous remains the bone must not be washed - else the DNA is prone to be washed out or being contaminated.

Once in the lab the samples should be stored under suitable conditions such as deep-freezing for moist samples or dry, remote and cool storage for dry or almost dry samples. The later recommendation is also true for samples origination from museum collections or recoveries previously done without considering aDNA analysis.

SAMPLE PREPARATION

Providing a DNA-Free Environment

As soon as the samples are under custody of the DNA expert, he or she has to ensure that no further decay can occur and no contamination-prone situation arises for the materials of interest. All tubes, buffers, containments and tools with the potential of sample contact must be purchased DNA free. Metal items such as grinding tools, forceps mortars etc. as well as glassware must undergo a dedicated process of DNA-decontamination, i.e. treatment with an alkaline detergent in a laboratory dishwasher and subsequent heat sterilisation (approx. 48 hours, 200-250 °C). Working tables, instrument switches, pipette-handles or touch displays should be decontaminated with chlorine bleach (DNA removal) and wiped with 75% v/v Ethanol (removing remnants of chlorine bleach). Some authors recommend the application of UV irradiation for surface decontamination (16) - others did no see any decontaminating effects on biological stains except with 'naked DNA' - hence, lab decontamination with UV apparently affects only purified DNA contamination but not cellular remnants (15). However, the application of UV has proven to be highly useful when applied to single use lab consumables such as pipette tips or reaction tubes.

All lab staff have to wear mouth protection, lab coats or cover-alls and - most important: disposable gloves, of course when handling the samples but also when operating lab instruments or computer keyboards, cameras, etc. Touching lab equipment with unprotected skin is prone to leave cell trace materials that may be transferred to the samples under investigation.

Sample Cleaning and Surface Decontamination

This chapter mainly refers to the author's own experience.

Regardless of the circumstances of sampling or origin of samples the first step in sample processing is the cleaning process: Use tooth brushes or similar and tweezers to remove soil, floral remnants or any other adherent originating from diagenetic or taphonomic effects. Remaining soft tissue should be removed with a sterile scalpel blade.

The next step can be assigned as surface decontamination / removal: Larger samples such as long bones, skulls or larger skull fragments, ribs, etc. have shown to be most efficiently processed utilizing a rotating wire brush or a sanding device to remove the outer surface layer given, that these objects offer enough space for a good grip. Smaller samples such as hand or finger bones, teeth etc. usually do not offer sufficient space to place a strong forceps or similar to grip for wire brush or sanding treatment. In these cases we successfully tested a procedure as follows: soak the sample (in a DNA-free

tube) in 75 % v/v of Ethanol followed by 15 minutes ultrasonic treatment, shake briefly, discard the liquid with removed particles; repeat this step with another portion of 75 % Ethanol followed by another ultrasonification step in 100 % Ethanol, finally discard the liquid as quantitatively as possible but take care not to loose the sample. Adherent and hence, potentially contaminating remnants are supposed to be physically removed from the sample by ultrasonification and up taken in the liquid provided. Use sufficient amounts of liquid - i.e. 30 ml of solution for a tooth or finger bone, approx. 100 ml for a talus or lager bone fragment respectively.

Finally, air-dry the sample remaining in the initial tube without lid - we recommend a dedicated DNA free incubator without air circulation (mandatory, risk of air borne contamination) - at approx. 60°C for 2-3 hours or more if necessary. The final treatment with 100% Ethanol and subsequent drying should remove the major amount of water from the sample (and hence, supporting the drying process) and prepare it for further processing. The 'washing' procedure in the lab - in contrary to the washing on the excavation site or in the museum or any other location than a dedicated lab environment - provides a DNA-free washing solution simultaneously avoiding dissolution and loss of DNA since a concentration of approx. 66 % of Ethanol in aqueous solution leads to DNA precipitation.

As indicated above, the utilisation of UV irradiation for surface decontamination appears not feasible for several reasons: Cells and cell fragments are obviously almost not affected, layers of surface adherent protect underlying contamination but most of all, as soon as there is no plain or even sample surface, naturally there are structures such as caverns, small caves / cavities or similar areas inaccessible for UV beams - i.e. anatomically restricted areas such as the space between the roots of a tooth, cracks or scratches in the sample surface, etc.

DNA ISOLATION and PURIFICATION

Physical Breakup

Larger bones or bone fragments can be further processed immediately after physical surface cleaning and decontamination: A device comprising of a box with openings for the hands containing a fixed funnel attached to vacuum device (we recommend a regular domestic vacuum cleaner) or any other suitable arrangement can be used for grinding the sample material with a drill, a milling cutter or a trepan. Place a filter paper in the funnel, operate the vacuum device and collect the bone powder in the funnel. This setting has been presented at a forensic conference in Salzburg in 2008 (17) and recently published by a french group (18). To our experience only powder from the bone compacta (but not from the spongiosa) has yieldes successfully type able samples - hence, as soon as the drill breaks through the compacta layer stop and move on in the compacta. Avoid collecting spongiosa material to minimize unintentional rarefaction of target molecules in the sample powder. After accomplishing the grinding process larger bits or fragments as well as soil or other particles not suitable for DNA extraction can be removed from the filter with a DNA-free forceps or any other suitable tool.

Be aware, that the bone powder frequently shows electrostatic effects due to the grinding/drilling process and may hamper transfer to extraction tubes. Finally take the filter from the funnel, collect the bone powder in the bottom by gently agitating the paper, rip off the upper brim and 'pour' the powder into a storage tube (i.e. 15 ml 'blue cap tubes' by Greiner), try not to touch the rim position where the powder is intended to leave the filter. The resulting bone powder can be used for chemical break-up without further treatment.

Smaller bone fragments (up to approx. 2 cm), small bones such as finger bones, metacarpals, or toes as well as teeth have to undergo a coarse break up i.e. by utilizing a mortar, a so-called 'bone gun' or any other suitable device to prepare it for grinding in a pebble mill at room temperature (we recommend stainless steel milling cups operated with a single milling ball). Alternatively a nitrogen-operated grinder can be employed, that does not require a pre-grinding break-up. Be aware, that the milling process warms the sample. Do not operate the mill longer than 60-90 seconds, check the temperature by touching the milling cup, and provide cooling breaks if necessary. In case the fine break-up was not sufficient after the first milling step, repeat the step one or twice to produce a fine mealy consistency. In case the drying process as described above was not sufficient, the cup's content shows a paste-like consistency – in this case add another 20-30 min in the drying incubator with lid open followed by a brief treatment in the mill to provide powder. Turning and tipping the milling cup against the target vial one can easily collect the resulting bone powder in a storage tube.

In some rare cases i.e. waterlogged bodies or vigorous purification under hot and humid conditions, major amounts of soft tissue are present but not suitable for DNA typing (according to our own experience). In these cases

we recommend a 10-15 cm section of the humoral or femoral diaphysis for DNA sampling. The application of a regular drill on the section's compata-parts producing tissue 'flakes' is recommended: The flakes are suitable for immediate chemical break-up.

Finally the authors want to state, that any procedure that is suited for the removal of superficial remnants, surface decontamination and sufficient physical break-up can be applied to hard tissues for the preparation of further chemical processing and DNA extraction, as long as a DNA-free non contamination environment is provided. Some studies indicate, that the fineness of the powder has influence on the quality of the DNA extracted, i.e.(19). Generally, we recommend dedicated instruments for aDNA only, however, in some laboratories, this is not feasible: Be aware of the contamination risks, apply precaution measures such as cleaning with chlorine bleach or similar.

Chemical Break-Up

Microanatomically, the DNA is located in the nucleus and the mitochondria and associated to proteins (histones). In contrary to soft tissues, the osseous matrix comprises of solid, mainly inorganic areas with caverns containing the cellular fraction (osteocyctes) and the DNA, respectively. Additionally, in dried bones with a long PMI (> years), the cells have undergone 'individual natural' mummification; the organic part appears to be associated ('touch dried') to the inner surface of the individual caverns(8).

The grinding and milling process allows access to the desired molecules at a microscopic level but the DNA is still associated with the matrix and other organic residues. These preconditions require a two-step treatment as follows: Decalcification dissolves the inorganic matrix mainly comprising of calcium and magnesium carbonates by immerging the bone / tooth powder in a buffer containing EDTA (ethylendiamintetra acid), a chelating reagent smoothly disintegrating the matrix. At the same time, the neutral or slightly alkaline pH value (7 - 8,5) and presence of EDTA ensures DNA protection from further alterations and decay mediated by DNAses. (EDTA binds essential co-factors such as Ca^{++} or Mg^{++} for DNAses.).

A subsequent step involving ProteinaseK and an adjuvant DTT (Dithithreitol) dissipates the proteins and ensures release of DNA to the aqueous solvent.

In detail, we recommend a procedure as follows: Taken into account, that each handling step and each additional reagent bears the risk of contamination, we focussed on a protocol employing as few steps and compounds as possible:

Transfer between 50 and 250 mg of bone / tooth powder into a 2 ml screw-cap vial by twisting the source vial (or storage vial, as described above) against the opening of the target tube. Do not use the 'regular' tubes with conic bottom since the bone powder tends to form a block in the conus, which has shown to be hard to suspend in the extraction buffer. Screw capped tubes are recommended since the decalcification / ProteinaseK-step requires 2-3 day of incubation under elevated temperature (56°C, permanent agitation); the lid-attached tube, even if purchased with a safe-lock lid tend to leak.

Add a suitable volume of 0,5 M buffered EDTA solution (depending on the protocol and the amount of bone powder available), ensure, that the powder is completely suspended (vortexing, snipping) and keep horizontal to avoid sedimentation until the samples go to the incubator.

Decalcification and subsequent ProteinaseK-treatment have to be performed under permanent agitation or rotation (along the roll axis of the tubes). Depending on the protocol, incubation duration between a few and up to 48 hours is recommended. Most protocols conduct partial decalcification – special protocols for low template samples (minute amounts of DNA) however refer to 'total demineralisation' in order to yield the maximum DNA available in the sample. The later ones naturally bear the elevated risk of contamination due to multiple steps during the decalcification process; additionally these protocols appear to be quite laborious but are recommended for samples of special interest or importance.

Most protocols recommend 56°C for decalcification / demineralisation – so do the authors, since a slightly elevated temperature should accelerate the reaction. Nevertheless the authors could not see a significant difference in DNA yield and quality of the results when comparing a small set of samples by incubating at room temperature (22°C), 30°C and 56°C.

After the demineralisation process a ProteinaseK-treatment has to be done to remove attached proteins from the DNA and release it to the aqueous solution. Substances such as DTT may be added as an adjuvant, the reaction can be boosted after a few hours to maximize DNA yield. For ProteinaseK incubation at 56°C is mandatory due to the reaction optimum of the respective enzyme.

Due to the authors experience the chemical break-up should be done as follows: Take between 50 and 250 mg

of bone or tooth powder, ad 650 µl of 0,5 M EDTA solution, pH 8,0; suspend the powder and place in a suitable incubator or agitation device as outlined above. Ad ProteinaseK (10-20 µl, approx 20 mg/ml) and optionally, the same volume of DTT (0,39 M), mix thoroughly and incubate at 56°C for at least 3-4 hours to overnight. Another optional booster-step (10 µl ProteinaseK) may be added. Make sure, the ProteinaseK is distributed evenly within the solution evenly (i.e. by stirring with the pipette tip) before adding the DTT since direct contact of both concentrated reagents is prone to reduce or eliminate enzyme activity due to the denaturising nature of the adjuvant. Low amounts of bone powder may result in total demineralisation but usually, a significant amount of remnant powder is found after completing the process as outlined above. A brief centrifugation step collects insoluble particles and provides a clear, particle-free supernatant, ready for further processing:

DNA PURIFICATION

In general this step should be as quantitative as possible since the 'usual' bone sample with a longer PMI has shown to contain only minute amounts of target DNA molecules. At the same time the purification process has to be very clean and restricted to DNA, since the raw extracts usually contain significant amounts of so called 'co purifying inhibitory substances', comparatively small organic molecules such as humic acids in case of soil born samples or other usually colourful yellow-brownish components. Additionally the aqueous solution containing the DNA contains inorganic compounds from the sample as well as a high amount of EDTA. The next step (polymerase chain reaction) is enzyme based, hence, all co purifying inhibitory substances have to be removed quantitatively since inorganic water soluble compounds distort the buffer system required for enzymatic activity, EDTA inhibits the polymerase due to its binding capacity of bivalent cat ions and humic acids and / or similar substances can cause inhibition due to their chaotropic activity.

Depending on the lab instrumentation equipment a semi-automated DNA extraction protocol is recommended. If no such device is available, suitable hands-on methods are available for DNA purification from ancient and forensic bone sample raw extracts.

When screening the literature on DNA purification methodology from bone and teeth extracts a common sense on sample pre-treatment, surface decontamination, physical and chemical break-up can be observed. However, the methodology of DNA purification can be subdivided into several groups, depending on the basic chemo-physical principle. Unfortunately the authors cannot give a recommendation on which protocols are most suitable since laboratories routinely performing DNA purification from bone and tooth extracts have established methods based on the availability of technical (financial) resources and know how dependent on the samples under investigation. Based on limiting factors each group of methods has its particular (dis)advantages:

Organic Extraction (i.e.(20))

These papers describe a procedure involving organic solvents (Phenol Chloroform Isoamylalcohol) for the extraction of organic compounds followed by a precipitation step to yield pure and PCR-suited DNA in aqueous solution. Organic extraction can be done without any further technical equipment as usually available in a forensic DNA laboratory and appears to be comparatively cheap. Organic solvents such as PCI are hazardous; the DNA-purification usually is a multiple step protocol with a comparatively high risk of contamination. Due to the author's experience, organic extraction followed by ethanol precipitation produces a good yield of DNA, however, some unwanted compounds might co-extract and have to be removed with another downstream protocol. Some raw extracts have shown to be not suitable for organic extraction since the aqueous phase turned into a jelly condition after adding the PCI; this may be due to the high concentration of a great variety of compounds leading to solidification. This problem can be overcome by diluting the raw extract.

Filtration Protocols (i.e.(21))

The raw extracts are applied to a filter device, flow through is mediated either by vacuum or gravity / centrifugation, followed by washing steps and elution in suitable solvent. These protocols appear to be easy and cost effective but also labour intensive and may require multiple washing steps. Unfortunately, many column or filter devices do no come with a lid. Performed under DNA free conditions these protocols have shown to be very good in DNA yield as well as in purity / removal of unwanted compounds, as recently shown in a collaborative study of 9 laboratories from Germany and Austria.

Batch Protocols (i.e.(22),(23))

Raw extracts are mixed with a suspension of glass beads; by altering the chemical conditions within the solution, the DNA is bound to the beads; after washing,

the beads with the DNA attached are physically isolated either by centrifugation (in hands-on protocols) or by application of an electromagnetic device in semi automated protocols. Aside of the tremendous acceleration semi automatation represents a significant improvement in DNA purification since the 'human factor' as a source of contamination and errors is (partly) suspended. These protocols share a significant disadvantage, since an expensive instrument with dedicated reagents and consumables is required. Another inconvenience may be a greater amount of bone powder required for extraction; however, the DNA yield and purity is comparatively good to excellent.

Alternative protocols: These protocols utilize an alternative principle for DNA purification, i.e. selective dilution of non-DNA compounds by a semi-permeable membrane (dialysis) followed by ethanol precipitation: This protocol has shown to share the same (dis)advantages as the filtration protocols as outlined above but is suitable for minute amounts of source materials (bone powder). In comparing the dialysis protocol with a semi automated one (QIAGEN M48) a comparable yield and quality of ready for PCR DNA was achieved by utilizing 50 mg for the dialysis-protocol versus 250 mg of bone powder for the semi automated one respectively.

DNA QUANTIFICATION

Many forensic DNA labs employ DNA quantification prior to PCR. First generation DNA quantification is based on fluorometry: The detection limit usually is at approximately 2-3 ng total DNA per µl. This method has proven to be extremely useful in 'regular' forensic case works, especially with samples with an expected high yield of DNA i.e. swab sample from sexual assaults or tissue from putrefied bodies, since these samples do not allow standardisation prior to DNA extraction (in contrary to buccal swab samples or blood samples). PCR systems like commercial multiplex kits come with a minimum/maximum recommendation of DNA input per reaction (usually between 0,5 and 5 ng of total DNA per reaction) at a certain number of cycles. According to the authors experience fluorometric DNA quantification of DNA extracts from ancient materials is useless since this method is not sequence or species specific. Due to taphonomic and diagenetic effects, the total DNA extracted most likely originates from microbes but not from the individual under investigation.

Second generation of DNA quantification employs real-time PCR systems (i.e. (24)): one or more dedicated DNA-target sequences are amplified. Successful amplification correlates with the emission of light at a certain wavelength, hence the PCR process can be monitored 'online' by direct detection of light and transformed into a measure for the amount of DNA present at a certain stage (cycle) during the PCR and is referred to the initial amount of target DNA in the reaction. Including an internal standard and one or more positive controls allow to ad data on mixtures (i.e. female versus male) as well as on the presence of PCR inhibitors. Moreover, the detection limit of real time PCR based quantification methods shows a detection limit several magnitudes below the fluorometric principle as outlined above – theoretically down to one single intact target sequence or molecule (the 'ultimate' detection limit). The read-out from the real time PCR serves as a tool for the assignment of DNA input and number of cycles for the subsequent multiplex STR (short tandem repeat profile or 'DNA fingerprint') or mitochondrial DNA targeted PCR.

Due to the authors' experience it remains discuss worthy whether or not real time PCR based quantification should be applied, since the limiting factor in ancient DNA analysis usually is the sample material. However, in case the lab has a dedicated procedure involving real-time data for the experimental design of downstream analyses, this 'intermediate' step appears to be highly useful, since the expected readout can be maximized by optimized reactions conditions. The use of real-time date to decide which sample is widely applied in forensics to eliminate non-promising samples; apparently (25), this is not always reliable – hence it is up to the individual researcher to include the real-time data for sample selection. Anyway, a systematic record of real time PCR based DNA quantities and presence of inhibitors in ancient DNA analysis represents a valuable amendment!

DNA TYPING and READ-OUT

After DNA extraction / purification and (optional) real time PCR-based quantification the following major options for information read-out – depending on the question - can be summarized as follows:

Sex Identification (i.e.(26),(27))

Especially in the context of molecular archaeological investigations, the identification of the biological sex is a crucial issue for den confirmation (or falsification (!)) of the anthropological and archaeological readout. DNA based sex identification is the method of choice in any case of hampered or impossible morphological sexing

(i.e. with infantile or juvenile individuals, missing body parts or highly fragmented remains) or in cases the archaeological assignment of gender is not concordant with the morphological finding. Most commercially available multiplex PCR kits include the Amelogenin-marker showing a sexual length dimorphism. In rare cases of failiure, the amelogenin-based test should be amended by additional markers such as the y-chromosomal SRY gene or lineage marker-kits (see below: chapter 'Lineage Markers'). In some cases, the application of single- or oligo-plex sexing PCRs may be inevitable, however, if solely applied there is no chance to proof data authenticity, as usually done utilizing autosomal multiplex kits (see below: chapter 'Data Authentication and Quality Control').

Autosomal Length Polymorphisms (STRs)

The assessment of an individual so called DNA-fingerprint represents a central read-out in forensic as well as archaeological casework. These kits simultaneously amplify approx. 17 or more autosomal non-coding markers characterized by length polymorphisms. Most kits available for forensics can be adapted for ancient DNA analyses by simply adding some cycles to the manufacturers' recommendation (i.e. from 30 to 34 cycles, resulting in an estimated elevation of sensitivity of approx. 10-100fold). Since multiplex kits are generally optimized up to a certain number of cycles (28-32) too many cycles lead to peak imbalance and artefact formation, some authors suggest to enhance sensitivity by elongation of the annealing and polymerization within a PCR-cycle or simply adding some units of polymerase (in case the enzyme included come in a separate vial within the kit). Extensive validation studies on recently released kits have shown, that so called 'fast protocols' designed for (direct) amplification or highly standardized buccal swab (saliva) or blood samples are not suitable for case work or ancient DNA samples likely due to reduced incubation times during the PCR process – hence we seriously recommend to maintain the 'standard' protocol including a final extension step between 45 and 60 minutes. This is especially important for the correct allel calling in cases of a single base pair difference in length since the Taq-polymerases usually utilized for these kits show a 'plus-A' activity (adding a singe adenin to every amplicon). This effect may result in a false heterocygous genotype in cases the final extension is long enough to make sure that every amplicon is completed with a final 'plus-A'.

In molecular archaeology, autosomal DNA fingerprints primarily serve as a tool for data authentication (28) (see below), assignment of single skeletal elements / individualisation within multiple inhumations (29), personal identification (i.e. historical persons (30)), assignment of family kinship (referring to the principles of paternity testing i.e. (31) or (32)) as well as for gross ethno-geographical estimation.

X/Y-Chromosomal (gonosomal) Length Polymorphisms (Y-STRs)

STRs or length polymorphisms are not restricted to autosomal chromosomes. Gonososmes, the X- and Y-chromosomes show the same short, tandemly and variably repeated structure and occur in noncoding regions of these chromosomes. Female individuals inherit one X chromosome from their mother and one from the father as observed with autosomes. In contrary, in (human) males, the X-chromosome always comes from the mother and the Y from the father non-recombined, unaltered and exclusively!

X-located STRs serve as an excellent tool in case of deficiency paternity (or maternity) cases or in the course of identification of unknown deceased based on family reference samples. As seen by forensics, X-markers usually are utilized to amend pre-existing results to consolidate the biostatistics. However, in ancient DNA analyses X-markers appear not to be used that frequently for some reason(s)(33).

Y-STRs / paternal lineage markers are widely used in forensics as well as in molecular archaeology for the assessment of male (perpetrator) DNA in sexual assault cases, in cases of human identification as well as in selected paternity cases assuming an unbroken paternal line. Moreover, recent authors experiences in forensic case works have shown that Y-STRs are suitable for intelligence databasing cases without sexual offences. Primarily, Y-STRs serve as an additional - in some cases a decisive tool in forensic and identification case works. According to the authors' experience, commercially available Y-STR-kits usually are adaptable for molecular archaeology purposes analogously to autosomal kits as described above.

Secondly, as seen from an anthropological point of view, Y-haplotypes represent a powerful tool for ethno-geographical estimations. Based on www.yhrd.org an unknown Y-haplotype can be assigned to the most frequent and the closest match within this database; hence, a statement on frequency and occurrence can be made referring to heritage, migration and residence of individuals and groups. CAVE: In ancient DNA studies

results have to be interpreted very cautiously since there is a temporal gap between the individuals under investigation (historical) and the reference database (recent). This has to be taken into consideration in provenancing ancient haplotypes. Due to the lack of recombination, Y-STRs are inherited comparatively conservatively and therefore are well suited for ethno-geographical estimation compared to autosomal markers (see above).

Mitochondrial DNA Sequences (mtDNA)

mtDNA reveals significant differences in analysis and interpretation: In contrary to STR analysis (length fragment analysis, length polymorphisms), mtDNA-read-out is based on DNA-sequencing, hence haplotype assignment refers (mainly) to sequence polymorphisms. In humans the mother inherits mitochondrial DNA information exclusively to all children. Therefore mtDNA – similar to Y-chromosomal haplotypes – can be used for the assessment of matrilineages as well as for ethno-geographical estimation at www.empop.org. Due to the comparatively low power of discrimination and other technical difficulties, mtDNA in forensic case works is only used in exceptional cases, i.e. when no nuclear / autosomal DNA can be detected due to degradation or extremely low amounts of target DNA or sometimes with hair samples. Based on the fundamental biological features, mtDNA occurs in a several magnitudes higher number of copies (several 100-1000) than nuclear DNA (2 copies per nucleus/cell), thus mtDNA analysis is successful in many cases no or only minute amounts of nuclear DNA can be (reliably) detected. This is especially true for identification purposes – and of course – for ancient DNA research.

Single Nucleotide Polymorphisms (SNPs) (i.e.(34))

Aside of sequence variations and length polymorphisms so called biallelic markers or SNPs represent another category of access to human DNA variation. A certain locus i.e. shows an A(denin) on a designated position in the genome with one part of the population and a C(ystein) with the other part. Analogously to the respective ways of inheritance, SNPs are handed down from one generation to the next and can be utilized for forensic case works, paternity testing, human identification and – of course – for ancient DNA studies. As indicated above, SNPs usually occur in 2 variants (bi-allelic), therefore these markers show a comparatively very low power of discrimination. Hence, many more single loci have to be characterized to achieve the same reliability as seen with STRs. Nevertheless, in some cases, SNPs are the method of choice, especially, in cases of highly degraded and/or fragmented DNA, since the length of the targets is – naturally - only one base pair. Moreover, SNP-typing can be done with different methods such as PCR based capillary electrophoresis, pyro-sequencing, real-time PCR or especially designed PCR primers referring to a match/mismatch situation during the annealing process with a binary result. Generally, SNPs allow assessing the same questions as mtDNA, autosomal and gonosomal STRs, (identification, kinship testing, ethno-geographical estimation) but with different information targets. Additionally, SNPs are often correlated with certain phenotypes or hereditary diseases (see below). When reviewing current literature, biallelic markers are not a mainstream method but appear to be extremely useful in challenging cases, whenever special DNA information readout is required or other well established and widely used methods do not supply satisfying results.

Phenotypical Markers (i.e.(35),(36))

Recent developments in forensics and molecular anthropology have demonstrated several novel assessments of phenotypical individual features such as hair, skin or eye colour and beyond. There is no doubt on the usefulness for palaeoanthropology in remains highly discussion worthy, whether the information is to be used for forensic case works due to the flawed reliability of the results (i.e. a crime scene trace originates with 70 % from a light skinned, blue eyed blonde person). Technically seen, the assessment of phenotypical features usually can be done by SNP-typing (as described above). A combination of several individual SNP-genotypes results in a good estimate of the correlating phenotype. Generally, complex phenotypes as mentioned above are not linked to a single or few genes or genotypes but the result of underlying genetic information is correlated with concrete SNPs.

Pathological Markers / Hereditary Diseases and Beyond

As soon as nuclear and/or mitochondrial DNA can be detected, the whole panel of known hereditary diseases is available, depending on the location of the corresponding genetic information. Human genome studies have shown, that hereditary diseases (disadvantageous wild type variations – most popular in ancient DNA studies the Russian heir to the throne Alexei Nikolajevitch Romanov (30)) are not only associated with variations in coding regions but also with neighbouring non-coding DNA loci due to a mechanism called 'epistasis linked loci'.

However, the prevalence of certain hereditary disorders such as sickle cell anaemia (37) or hemochromatosis (38) in present and – especially in ancient populations represents not only valuable information on the affected individuals but also on environmental interactions with the individual or population under investigation. In some cases, pathological findings on the osseous remains can be confirmed or cross-validated by ancient DNA analysis.

Beyond pathological variations the assessment of metabolic enzymes such as lactase (39) represents another extremely valuable tool for studying ancient and prehistoric civilisations.

Pathological Markers / Infectious Diseases

In contrary to the previous chapter, infectious diseases can be detected by finding the pathogens' DNA with or in the remains under investigation: This might be useful in forensics and pathology, in cases of contagious diseases endangering the pathologists but the main focus lies on the study of ancient individuals and civilisations. The assessment of 'scourges of mankind' not only provides extremely valuable information on everyday life in past populations, politically decisive epidemics such as The Great Plague (40) but also on the origin and development of currently ongoing diseases involving pharmacological research. As seen from the focus of ancient DNA / palaopathology, the preservation of pathogen DNA on or within the remaining tissue is crucial. Since soft tissue is preserved only in the minority of historical cases, however, i.e. in the course of investigation the Spanish Flue from 1918 (41) lung tissue from victims was the sample of choice. Aside of these rare cases, infections affecting the bone such as tuberculosis (42) or syphilis (43) are accessible as well as plague or leprosy (44). Infections with a very high or very low lethality are less probably found in ancient human remains due to the fast diying with the first ones or pathogen clearance by immune system with the second ones. Additionally, the detection of pathogen species might be obstacled by cross reactions or contaminations from the soil or by other digenetic effects.

DATA AUTHENTICATION, QUALITY CONTROL and BIOSTATISTICS

The Four-step Model of Data Authentication and Quality Control

From the very beginnings of ancient DNA research and palaogenetics data authentication and quality control represents a central and crucial issue. Aside of logistic measures such as providing a DNA-free environment, temporal and spatial separation of pre- and post-PCR works etc. we propose a 4 step-model of data authentication(12):

Step 1: All blanks and negative controls must not show any detectable DNA, positve control must show the expected result.

Step 2: Successfully typed ancient or historical samples have to mismatch the DNA profiles of all affiliated persons (potential contaminators).

Step 3 (optional): confirmation analysis by another dedicated laboratory; however step 3 is limited due to financial, technical, personal as well as sample resources. In general validity (the method employed has to be suited to answer the question(s)), reliability (independent replication of the analyses lead to the same result) and objectivity (independent replication of testing by different persons in different laboratories leads to the same result) have to be assured as far as possible!

Step 4 (optional): Even in cases Step 1-3 are completely fulfilled, there are still imponderables such as unknown / unidentified sample contamination, historical contaminations (i.e. in cases of museum specimens or samples from old collections or previously excavated collections). Hence, one can never be 100 % sure on the authenticity of the results but one can get pretty close to: The proof of ancient family (parental) kinship within a finding or tomb/grave is a final, extremely strong indicator for authentic DNA data.

Another good indicator for data authenticity is the detection of DNA degradation (i.e. a slope in signal height versus fragment length) since modern contamination(s) usually show good peak balance across the whole DNA pattern.

Further Considerations

In human identification a DNA profile (composite of STR-markers) or a mitochondrial DNA sequence is compared to a reference sample – in case of a match positive identification achieved with minute uncertainties: Regardless whether the questionnaire comes from regular forensics of from the historians the reference sample of choice is a 'direct comparison sample': anything from a toothbrush to worn clothes may provide biological remnants from the user or owner; in case of a match, it appears highly likely (approx. $1:10^{20}$) that the person under investigation has left its biological material on the reference trace sample versus a random match. However, as soon as the PMI exceeds a few weeks to months, these samples tend to become rare or rather contaminated by other persons, especially in historic case

works. In these cases one strives to get a sample from a living relative, especially focussing on the biological mother ('*mater semper certa est'*) or in case the deceased is female on children, if available. Other settings of family kinship are suited as well but are linked to some uncertainties, since the 'social' father not necessarily is the biological one. Due to meiotic recombination, siblings are not as well suited as parents or children since these cases require extended genotyping with a large panel of markers (i.e. including X/Y-STRs) to achieve the same biostatistical secureness. Transgenerational lineage markers without meiotic recombination such as mtDNA and Y-chromosomal haplotypes are the analytical targets of choice in case one or more generation lye between the unknown individual of interest and the reference sample (donor). Biostatistical evaluation of the results is an absolutely critical step: In simple, uncomplicated constellations (i.e. as seen in paternity testing) the situation is comparably easy: A mismatch of DNA profiles is 100% exclusive, a match usually reveals 99,99% or more probability of parentage versus a random match. The situation is different in mtDNA (matrilineage) since the power of discrimination is far beyond nuclear DNA, as indicated above. At some degree a match in Y-haplotypes comes with the same imponderabilities but less severe. The number of meioses between the reference sample and the individual to be identified appears to be the crucial factor. Hence, apart from discontinuities in the maternal or paternal line, a mismatch does not necessarily mean that there is no kinship or vice versa. Mutational events have to be taken into biostatistical consideration when assigning a final statement on the probability of kinship or positive identification respectively, especially in analyzing remains of historical persons involving living sample donors with reputed biological kinship!

Outlook – NGS – Next Generation Sequencing

Recent developments lead to the so-called 'next generation sequencing' or NGS-technology (36). This method still is based on purified DNA as outlined above but does not utilize PCR and capillary electrophoresis. Sample DNA is processed to establish a library of molecules, which serves as a basis for reading the complete genetic information from the DNA extracted. Due to the comparably high price of the instruments, reagents and other consumables and the challenging data read and interpretation, this technology is not yet routinely applied, neither in forensics nor in ancient DNA / palaeogenetics. However, as soon as a DNA-library, that theoretically allows reading a whole (human) genome (or any DNA encoded information from a certain sample) has been established, this technology appears to be the most promising innovation in the field of DNA trace analysis.

Concluding Remarks

Synoptic evaluation: Despite interfering and restraining factor and events such as DNA-degradation or contamination reading the DNA molecule has proven to be an extremely powerful tool in forensics as well in history and archaeology. In any case, the synoptic evaluation of all findings is essential to evaluate a case. Casually the DNA results are decisive on a case but have to be interpreted and valued together with all other involved experts to establish a final record including all known and unknown imponderables.

Ethical considerations: According to the authors conviction genetic information underlies the owners / donors 'copyright' – therefore we recommend an assurance of confidentiality by a written paper. In ancient DNA studies, a set of reference samples for data authentication is crucial, as outlined above. If a potential contaminator is not willing to give a sample we have to respect that – however the person in charge of the project may draw the obvious conclusion.

Respectful treatment of human remains represents another central issue as well as religious or other ethical concerns i.e. by living relatives or other persons or institutions involved. Especially when it comes to excavations or exhumations in the conduct of historio-archaeological research, approval of all affiliated persons and institutions is a mandatory prerequisite.

Conflict of interest

None.

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