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General Palaeontology (Taphonomy and Fossilisation)

Environment and excavation: Pre-lab impacts on ancient DNA analyses

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Abstract

Ancient DNA (aDNA) analyses enjoy an increasing role in palaeontological, archaeological and archaeozoological research. The limiting factor for aDNA studies is the degree of DNA preservation. Our study on 291 prehistoric cattle remains from Europe, the Near East and North Africa revealed that DNA preservation is mainly influenced by geographic and climatic conditions. Especially in hot climates, the preservation of sample material is generally low. We observed that these specimens are prone to further degradation and contamination during and after excavation. We give a description of the main caveats and a short guideline for adequate sample handling in order to facilitate the cooperation between archaeologists and geneticists and to improve the outcome of future research. ***To cite this article: R. Bollongino et al., C. R. Palevol 7 (2008).***

Résumé

Influence des conditions environnementales et de la fouille sur l'analyse de l'ADN ancien. L'ADN ancien joue un rôle de plus en plus important dans les recherches paléontologiques, archéologiques et archéozoologiques. Le degré de préservation de l'ADN est toutefois un facteur limitant. Les données que nous avons réunies sur 291 restes de bovins préhistoriques d'Europe, du Proche-Orient et d'Afrique du Nord indiquent que la conservation de l'ADN est principalement influencée par les conditions géographiques et climatiques. Plus précisément, la conservation est généralement mauvaise dans les zones au climat chaud. Nous avons observé que ces spécimens sont sujets à des dégradations et contaminations pendant et après la fouille. Nous donnons ici une description des principaux biais ainsi que quelques recommandations pour le prélèvement des échantillons, afin de faciliter la coopération entre archéologues et généticiens et d'améliorer l'efficacité des recherches futures. ***Pour citer cet article : R. Bollongino et al., C. R. Palevol 7 (2008).***

Keywords: Ancient DNA; Contamination; DNA preservation; Sampling strategy; Molecular genetics; Excavation techniques

Mots clés : ADN ancien ; Contamination ; Préservation de l'ADN ; Stratégie d'échantillonnage ; Génétique moléculaire ; Techniques de fouille

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1. Introduction

Despite the unquestionable progress and increasing importance of ancient DNA research, studies that yielded no results are rarely published, leaving the palaeontologist and archaeologist unaware of possible caveats. Ancient DNA analyses are limited by a simple factor: the preservation of DNA. Bone diagenesis and degradation of biomolecules is a complex field that is not yet understood in all aspects. Nevertheless, the main factors can be summed up as follows [6,8–11].

Water is a main degradative factor; it dissolves the bone apatite, allows growth of microorganisms and leads to hydrolytic and oxidative damages of the DNA. Microorganisms (bacteria and fungi) metabolize the organic components of the bone, such as collagen and DNA. Increased temperature enhances chemical decomposition and proliferation of microorganisms. Sunlight (UV light) increases the amount of free radicals that lead to DNA oxidation. Acidic soils dissolve calcium phosphate and thus destroy bone apatite. Alkaline environments (e.g., limestone and karst formations) buffer the degradative effects of acids and stabilise bone apatite. On the other hand, bicarbonate emerges under alkaline conditions and the presence of carbon dioxide, which increases the degradation of bone apatite. The most stable state of hydroxyl apatite could be observed at a pH of 7.8 [10,11]. Thus, as a rule, DNA preservation is best under cool, dry, dark, anaerobic, and slightly alkaline conditions.

There is still no reliable screening method for DNA preservation, except the molecular genetic analysis itself. However, our studies strengthen previous observations (see Geigl's and Hofreiter's papers, this issue) that an appropriate sample treatment during and after excavation can have an important impact on DNA preservation and sample quality. Especially highly degraded specimens, as they are typical for hot climates, require particular precautions. The aim of this article is to improve the understanding of the interrelations between sample treatment and a successful molecular genetic analysis. Moreover, we give a short guideline for archaeologists and palaeontologists about adequate sample handling and storage.

2. Data basis

The phenomena described in this article are based on the analyses of mitochondrial DNA of 291 prehistoric cattle samples from Europe, the Near East and northern Africa (details are shown in Table 1) [2,4]. The samples were grouped by different climatic and geographic regions: central and western Europe, southeastern Europe, Near East and North Africa. In order to describe the DNA preservation of the samples, we subdivided the results into three groups: (1) samples that gave replicable results, (2) samples that gave non-replicable results, (3) samples that were not amplifiable. However, the amplification success is influenced by many factors such as genetic locus, fragment length, laboratory methods, and amplification protocol, etc. In order to keep the dataset manageable, it was not possible to consider all factors. Thus it should be kept in mind that the presented success rates are only approximations.

3. Impact of environment and excavation technique on sample quality

3.1. Environment and DNA preservation

The average amplification success rate of all 291 samples was 46%, while 8% gave DNA, but were not replicable (see Table 2). Forty-seven percent were not amplifiable at all. In general, we observed a north–south decline in DNA preservation for open-air sites, with 67% success rate in central Europe, dropping to 7% in the Near East.

Within Europe, there is a correlation between amplification success and soil type. Sites from calcareous soils such as Eilsleben, Roucadour, and Tai (see Table 1) show a success rate of more than 80%. In contrast, sites from low pH soils in southern Germany (Mitterfecking, Bad Abbach, Hilzingen) hardly revealed any DNA. This contrast is strengthened by the fact that numerous sites in calcareous contexts are karst sites, either caves or rock shelters (see below).

We could not observe a noticeable difference in DNA preservation between central Europe and southeastern Europe. Like for central Europe, small-scale regional differences in geography and climate seem to be crucial.

The least advantageous conditions for DNA preservation could be found in the Near East. The amplification success is similar to the one observed by Edwards and colleagues [3]. The combination of high temperatures and a relatively constant humidity below the surface foster a rapid bone diagenesis. The two samples that gave results from Dja'de and Tell Brak (Table 1) originate from deep layers, while samples that were closer to the surface gave no DNA. We assume that the structure of the Tell, which is often several meters high, protects the bones deep below to some extent. Compared to European samples, all Near Eastern samples were of a porous and soft structure and the few samples that yielded

Table 1
List of archaeological sites, number of samples and amplification success
Tableau 1
Liste des sites archéologiques, nombres d'échantillons et taux de réussite des amplifications

		Cave/rock shelter	No. of samples	+	+/−	−
Central & western Europe						
	Belgium					
	Goyet Cave	×	2	2	0	0
	France					
	Bercy		8	7	0	1
	Baume d'Oulen	×	2	2	0	0
	Charente River		1	1	0	0
	Cave à l'ours	×	1	1	0	0
	Combe obscure	×	5	5	0	0
	Er-Yoh		2	1	0	1
	Etival		1	1	0	0
	Grotte Champeau	×	2	0	0	2
	Grotte de la Bouloie	×	2	0	0	2
	Grotte du Gardon	×	1	1	0	0
	Igue du Gral	×	1	0	0	1
	Mareuil-les-Meaux		4	2	0	2
	Pont de Roque-Haute		1	0	0	1
	Roquefure	×	1	0	0	1
	Roucadour	×	8	6	2	0
	Ruffey-sur-Seille		8	1	0	7
	Grotte du Tai	×	5	5	0	0
	Trosly-Breuil		7	2	0	5
	Germany					
	Allendorf		1	1	0	0
	Bad Abbach		2	0	0	2
	Berlin-Köpenick		1	1	0	0
	Derenburg		1	1	0	0
	Eilsleben		12	10	0	2
	Goddelau		3	3	0	0
	Göttingen FMZ		3	0	0	3
	Halle		1	1	0	0
	Hilzingen		3	0	0	3
	Mitterfecking		1	0	0	1
	Neustadt (Schleswig-Holstein)		2	2	0	0
	Nieder-Mörlen		4	3	0	1
	Quenstedt		3	3	0	0
	Rosenhof		9	9	0	0
	Schwanfeld		1	0	0	1
	Siegsdorf		1	1	0	0
	Trebur		4	3	1	0
	Viesenhäuser Hof		8	3	1	4
	Wangels		7	7	0	0
	Italy					
	Isernia		1	0	0	1
	Sardinia					
	Cuccuri is Arrius	×	2	0	0	2
	The Netherlands					
	Emmeloord		1	1	0	0
	Switzerland					
	Château-d'Œx		1	1	0	0
Southeastern Europe	**Bulgaria**					
	Orlovez		2	0	0	2
	Hungary					
	Albertfalva		4	4	0	0
	Berettyószentmárton		6	1	1	4

Table 1 (*Continued*)

		Cave/rock shelter	No. of samples	+	+/−	−
	Budapest		2	2	0	0
	Herpaly House		2	0	0	2
	HódmezövásárhelyBod.		1	0	1	0
	HódmezövásárhelyGorza		2	1	0	1
	Polgár-Csöszhalom		5	3	0	2
	Szegvár-Tüzköves		2	2	0	0
	Rumania					
	Bordusani Popina		3	3	0	0
	Bucsani Pod		1	1	0	0
	Cheia		2	2	0	0
	Sardinia					
	Cuccuri is Arrius	×	2	0	0	2
	Slovenia					
	Svodin		3	3	0	0
	Slovakia					
	Ljubljana		3	3	0	0
	Thrace					
	Asagi Pinar		2	2	0	0
	Hocaçeşme		3	1	0	2
Near East						
	Cyprus					
	Shillourokambos		2	0	0	2
	Georgia					
	Didi Gora		3	1	1	1
	Israel					
	Abu Gosh		1	0	0	1
	Atlit Yam		1	0	0	1
	Kfar Hahoresh		1	0	0	1
	Lod NY		1	0	0	1
	Tell Hreiz		1	0	0	1
	Syria					
	Dja'de		10	1	0	9
	Haloula		1	0	0	1
	Tell Aswad		7	0	1	6
	Tell Brak		3	1	2	0
	Tell Qaramel		6	0	0	6
	Turkey (except Thrace)					
	Çatal Höyük		4	1	0	3
	Çayönü		7	0	2	5
	Fikirtepe		3	0	0	3
	Mezra Tel Eilat		4	0	0	4
North Africa						
	Morocco					
	Ifri el Amas	×	20	4	0	16
	Ifri el Baroud	×	15	2	2	11
	Ifri Oudadane	×	2	0	0	2
	El Harhoura	×	8	3	2	3
	Hassi Ouenzga	×	3	0	1	2
	Taghit Haddouch	×	9	1	5	3

The archaeological sites are grouped by geographic regions. + = samples that gave replicable results, +/− = samples that gave non-replicable results, − = samples that were not amplifiable.

Les sites archéologiques sont regroupés par grande région géographique. + = échantillon ayant livré un résultat répliquable ; +/− = échantillon ayant livré un résultat n'ayant pas pu être répliqué ; − = échantillon n'ayant pas pu être amplifié.

Table 2
Mean amplification success of samples grouped by geographic regions
Tableau 2
Taux de succès moyen des échantillons par région géographique

	n	+	+/−	−
Central & western Europe	136	67%	3%	30%
Eastern Europe	43	70%	4%	26%
Near East	55	7%	11%	82%
Morocco	57	17%	17%	65%
Σ	291	46%	86%	47%

Note that all data are just mean approximations; amplification success is, e.g., dependent on fragment length and protocol, details that were not taken into consideration. Central and western Europe: Belgium, France, Germany, Italy, The Netherlands, Switzerland. Southeastern Europe: Bulgaria, Hungary, Romania, Slovakia, Slovenia, Thrace. Near East: Cyprus, Georgia, Israel, Syria, Turkey (except Thrace). + = Samples that gave replicable results, +/− = samples that gave non-replicable results, − = samples that were not amplifiable.
Ces données ne sont que des approximations moyennes, le succès de l'amplification dépendant aussi, entre autres, de la longueur du fragment d'ADN choisi et du protocole mise en œuvre, qui n'ont pas été pris en considération ici. Europe centrale et occidentale : Allemagne, Belgique, France, Italie, Pays Bas, Suisse. Europe du Sud-Est : Bulgarie, Hongrie, Roumanie, Slovaquie, Slovénie, Thrace (Turquie). Proche-Orient : Chypre, Géorgie, Israël, Syrie, Turquie (sauf la Thrace). + = échantillon ayant livré un résultat répliquable ; +/− = échantillon ayant livré un résultat n'ayant pas pu être répliqué ; − = échantillon n'ayant pas pu être amplifié.

DNA were difficult to amplify, indicating an advanced degradation.

Exceptional good DNA preservation can be found in caves [5]. Caves usually have a stable low temperature all year-round and often offer a calcareous environment, which decreases the microbial activity and solubility of the bone apatite [10]. A lack of precipitation additionally protects the bones from degradation [6]. Though the majority of the central & western European cave samples were stored under unknown conditions for several years or decades, the average amplification success was 75% ($n = 16$, central European success rate for open-air sites is 65.8%, $n = 117$). The fact that the amplification success for Moroccan samples is 17.5% compared to 7.2% in the Near East is probably due to the fact that all Moroccan samples come from cave sites. However, the majority of the Moroccan samples only gave very short DNA fragments, thus indicating an advanced degradation compared to European caves.

The described factors have a major influence on the preservation of DNA. However, small-scale conditions can be the reason why some bones are much better preserved than others from the same site. It is for example known that metal objects that contain copper inhibit bacterial growth. The presence of a limestone or pottery next to a bone might protect bone apatite and buffer low pH values. Samples from a cave might be less well preserved when they lay close to the cave walls that are often covered with seepage water, or when flocks have been kept in the cave, bringing large quantities of excrements into the soil.

Contaminations can impede DNA analyses despite good sample preservation. Pre-laboratory sources of contamination will be presented in the following.

3.2. *Contaminations from the soil*

Within the Moroccan specimens, we observed a special case of contamination: though bones were recovered under clean conditions and not washed, some of the cattle samples were contaminated by goat DNA. The samples come from five different caves that are still used as a shelter for sheep and goat. The sediments contain a lot of excrements, contaminating the skeletal remains underneath. Approximately 19% ($n = 57$ samples) of the bones occasionally gave goat DNA. From one specimen both cattle and goat DNA was amplified at the same time.

Such severe contaminations impede a reliable ancient DNA analysis of sheep and goat. To spot such cases, which are often not as obviously detectable as in our case (e.g., if the use of the place for animal keeping goes back long ago), we suggest to take additional control samples to avoid false positive results (see § *Control samples*). However, well-preserved samples with an intact bone structure should not be contaminated underneath the bone surface.

3.3. *Post-excavation influences*

During the excavation, the environment of the specimens is suddenly changed. Especially in hot climates, they are often exposed to sunlight and heat for hours and suffer from increased thermal stress [14]. Touching sampling material without gloves will leave the DNA of the excavator on the surface of the specimen, which might subsequently be carried into the bone when the sample is being washed. Washing of samples is a ubiquitous practice in the field. It facilitates further examination, determination and measurement of the specimens, steps that are usually carried out *before* a genetic analysis is taken into consideration.

Tap water is not free of DNA and thus a serious source of contamination. Generally, water is used for several samples and we observed at one site that the same tub was even used as a watering place for sheep and goat. We monitored the temperature changes during sample washing on a Near Eastern excavation and observed a

severe temperature drop and subsequent increase, causing a significant thermal shock [1].

Usually, well-preserved samples will not be harmed by this treatment, as contaminations should remain on the bone surface, which can be decontaminated in the laboratory. For example, all samples from central and western Europe had been washed without leaving any detectable contamination, though no inferences can be made about a possible impact on DNA preservation.

However, especially in hot climates, many bones loose their dense and compact structure during diagenesis, leaving them vulnerable for contamination and loss of DNA. Washing enhances further degradation [6], but most notably, porous bone and teeth structures are penetrated completely by water, which carries contaminations deeply into the tissue. Molecules at the sample surface can be removed in the laboratory, but contaminants inside the sample will always be co-extracted. Three out of 32 samples from different excavations in the Near East yielded occasionally DNA from a different species, though the bones clearly originated from cattle. From 23 samples that were not washed after excavation, no goat contamination could be observed.

4. Conclusions and guidelines

We observed that, especially in hot climates like the Near East and North Africa, the excavation technique and storage of samples often fosters DNA degradation and increases the risk of irreversible contamination. Thus, an adequate treatment of bone specimens can be crucial for a subsequent analysis biomolecules.

In the following, we give some rough guidelines for ancient DNA sample handling. However, techniques are constantly improving and different projects might require different methods. Thus we recommend to consult a palaeogeneticist to clarify all details on sample choice and sample treatment prior to excavation. Even when no DNA analysis is planned, it is advantageous to treat samples adequately to allow future analyses of ancient biomolecules.

4.1. Choice of samples

It is very difficult to estimate the preservation of DNA by macroscopic inspection of the specimen. However, there are some features that are characteristic of good samples: bones should be hard, heavy and of a compact structure. Long bone diaphyses are preferable to porous, spongy bones like vertebra, scapulae, pelvis, and some part of the skull.

Bones should display only few or no cracks and microbial attack (visible as tiny black holes and lines). Especially for human specimens, teeth with well-preserved roots proved to be useful. Burnt or heated material is not suitable.

When sample size is large enough, the same anatomic elements should be picked to avoid double-typing of individuals. Furthermore, being included in archaeological items, aDNA molecules are themselves archaeological items. As such, samples for aDNA should come from a clear archaeological context and not from disturbed layers.

4.2. Sample excavation

Our data from the Near East and Morocco show that, especially in hot climates, samples are less well preserved and thus vulnerable to thermal stress and contamination during excavation.

Samples can be kept constantly under relatively cool temperatures when they are excavated quickly (if possible with gloves on), put directly into a clean bag and stored in a portable electric cool box [1] or at least in a shady place. If no electricity or cool places are available, the samples should be put into bags and burrowed into the ground, deep enough so that the heat of the day will not reach them. It is also important to keep domestic animals away from sample material. We observed that especially sheep and goat are common guests at excavations, leaving, e.g., hairs and excrements and thus a potential source of contamination.

Human remains are especially prone to contamination, as human-contaminants are seldom distinguishable from the authentic sample DNA. In order to keep the contamination risk as low as possible, excavators should at least wear gloves and facemasks. An additional smock or clean room suit and hair cover would be ideal. Tools, tables etc. can easily be decontaminated with bleach. Gloves should be changed after every sample handling in order to avoid interfossil contamination. For a genetic analysis, each sample will be extracted twice for authentication. Thus it proved to be useful to take two samples per individual, e.g. a long bone and a tooth. We recommend to consult a specialist of ancient DNA prior to the excavation to discuss an appropriate sampling strategy.

4.3. Sample treatment

Whenever possible, gloves should be worn for sample handling, especially for human material. Each specimen should be put in a separate plastic bag to avoid contaminations between them. In case samples are humid

and cannot be kept in a refrigerator, they should be dried carefully in a cool and shady place, covered by tissues.

We showed that washing samples is a severe source of non-removable contamination, additional thermal stress and degradation (see above paragraph "post-excavation influences"). We recommend not to wash samples at all. If osteometric measurements are necessary prior to DNA analysis, samples should just be cleaned with a brush.

4.4. Sample storage

Pruvost and colleagues [13] demonstrated the importance of storage conditions: they found no DNA in a specimen excavated 57 years ago, but successfully amplified DNA from a freshly excavated sample of the same bone, that had been accidentally split during the initial excavation.

To minimise the risk of (post-excavation) DNA degradation, samples have to be stored under cool, dry, and dark conditions. It is ideal to keep dry specimens in a refrigerator (or freezer, which is less favourable because shear forces of ice crystals might damage the DNA). In the field, there is often no possibility to store samples in a refrigerator. In hot climates, electric cool boxes proved to be a useful solution to protect specimens from the surrounding heat. If no electricity or cool places are available, plastic bags with dry samples (see paragraph above) can be buried deep enough to keep the heat of the day away.

For long-term storage, it is important to choose a place that is cool and dry during all seasons of the year.

4.5. Control samples

Our data showed that, in some cases, samples can be contaminated by the surrounding soil. A helpful means to monitor this contamination is additional control samples. Soil samples from different parts of the excavation should be collected and treated like samples (i.e. kept in a cool and dry place). As many soils contain substances that inhibit DNA analysis, additional bone samples of a different species than the one that is studied should be taken [5]. In case of human material, DNA of all people who handled the samples should be provided. This ensures that a possible contamination by archaeologists can be detected.

It is a common practice that ancient DNA results are replicated in a second lab [7,12]. To assure an independent replication and to rule out a contamination of the sample in the first lab, or to allow additional ^{14}C dating or isotope analysis, a sub-sample should be kept and stored at a place where no molecular work is carried out. Our practice is that the main sample (in general the articular parts of the bone) remains in the archaeological or archaeozoological laboratory, and that only sub-samples (diaphyses fragments) are sent to the molecular genetics laboratory. This also allows to keep the diagnostic anatomic part for further measurements and taxonomic verification.

4.6. Useful information for geneticists

As the conditions in the field as well as the archaeological context can be of great importance for the interpretation of the genetic data, it is advisable to provide the geneticist with relevant information. Basic data like archaeological period and age estimation should be completed by information about sample treatment, possible sources of contamination (e.g., presence of animals or unprotected handling of sample material), available ^{14}C dates, probability that different bones stem from the same individual, and storage time and conditions since excavation. Furthermore, osteometric measurements can be very helpful in case of ambiguous archaeozoological classifications, especially when discrepancy with the genetic results arises.

Ancient DNA studies have an increasing relevance in archaeological and archaeozoological fields. Unfortunately, historically important regions like the Near East, and, more generally, arid and semi-arid regions are characterised by inadequate conditions for DNA preservation. Conventional excavation techniques expose the samples to additional stress and thus diminish the chances for successful genetic analyses. The presented data just give a first impression of these problems and need to be completed by further research. However, we hope that this article will enhance the understanding of an adequate sample treatment to improve conditions for future studies. Close cooperation and information exchange between geneticists and archaeologists already proved to be fruitful in terms of increased sample quality and better understanding of the results.

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