

General Palaeontology (Taphonomy and Fossilisation)

Palaeogenetics of cattle domestication: Methodological challenges for the study of fossil bones preserved in the domestication centre in Southwest Asia

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Abstract

Recently, palaeogenetics encountered enormous success when parts of the nuclear genomes of mammoth and Neanderthal man were analysed. Their bones, however, had been preserved in environments favourable to DNA preservation, i.e., permafrost regions and caves in temperate regions. Few studies have tackled archaeological bones from hot, arid regions, although they bear great significance for the study of evolution of humans and the precursors of modern societies. According to archaeological evidence, a key event in neolithisation, the domestication of cattle, took place around 10,000 years ago in Southwest Asia. Genetic data from prehistoric bovine bones preserved in this region might shed light on this process, but the palaeogenetic approach has been hampered by poor DNA preservation. Here, I discuss various aspects of DNA preservation in fossils and the production of reliable palaeogenetic data and present methodological improvements that have enabled us to shed light on the process of cattle domestication in Southwest Asia and its spread into western Europe. **To cite this article:** *E.-M. Geigl, C. R. Palevol 7 (2008).*

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Résumé

Paléogénétique de la domestication des bovinés : défis méthodologiques posés par les ossements conservés dans le foyer de la domestication en Asie du Sud-Ouest. Les grandes avancées de la paléogénétique ont surtout été réalisées à partir d'os conservés dans des environnements favorables, comme le permafrost ou des grottes des régions tempérées, mais il n'existe que peu d'études réalisées sur des os archéologiques de régions chaudes et arides, qui présentent pourtant le plus d'intérêt pour l'analyse de l'évolution de l'homme ou des précurseurs des sociétés modernes. L'archéologie a montré qu'un événement clé de la néolithisation, la domestication des bovinés, s'est produit, il y a environ 10 000 ans, en Asie du Sud-Ouest. La compréhension de ce processus serait éclairée par l'obtention de données génétiques issues d'os préhistoriques bovins de cette région, mais une telle analyse paléogénétique s'est heurtée jusque-là à une mauvaise conservation de l'ADN. Je discute ici divers aspects de la conservation de l'ADN dans les fossiles et de la production de données paléogénétiques fiables ; je présente ensuite les améliorations méthodologiques qui nous ont permis d'éclairer le processus de la domestication des bovinés en Asie du Sud-Ouest ainsi que sa propagation en Europe de l'Ouest. **Pour citer cet article :** *E.-M. Geigl, C. R. Palevol 7 (2008).*

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1. Introduction

Palaeogenetics, the retrieval and analysis of genetic information from fossils, the only direct witnesses of extinct species and of evolutionary events, has afforded new insight into evolutionary biology and archaeology. Analysis of human fossil remains enriches our knowledge of the evolution of man, while the analysis of the remains of domesticated animals and plants adds to our understanding of the cultural evolution of humans during the Neolithic. Through analyses of the genetic diversity of domesticates, we also gain insight into the effects of human action on the environment.

Despite the enormous potential of palaeogenetic analyses and the considerable progress that has been achieved during the last years, there are constraints that are difficult to overcome. These limits are related to the post-mortem degradation of DNA. In fact, DNA in living cells is constantly degraded and altered by endogenous and exogenous factors, such as intracellular enzymes and radicals, UV light and mutagenic environmental chemicals, respectively. The resulting DNA damage is repaired by the enzymatic repair machinery of the cell. After the death of an organism, these repair processes come to an end, while the degradation processes continue and even increase. Whatever is left after autolysis, i.e., degradation of the cellular components by the cellular enzyme machinery itself, and initial putrefaction, i.e., degradation by microorganisms, will be subjected to chemical reactions such as hydrolysis and oxidation, which ultimately lead in the vast majority of cases to total degradation of DNA. In bones that undergo fossilisation and which I will hereafter call ‘fossil’ bones, DNA can sometimes escape total degradation and be preserved in extremely small quantities of small, damaged fragments – permafrost specimens being an exception, since they often contain relatively high quantities of DNA that are less degraded [78].

It is clear from a literature survey that palaeogenetic studies are mostly performed with specimens that are less than 100,000 years old and have been preserved under cold climatic conditions. These studies have led to some spectacular achievements, such as the sequencing of one million base pairs of DNA of *H. neanderthalensis* from a specimen preserved in a cave in Croatia [42] or 13 million base pairs of mammoth DNA preserved in the ice in Siberia [78]. A closer look at the geographical and temporal map, however, reveals that there is an inhomogeneous distribution in time and space of palaeogenetic studies of animal and human remains. While the permafrost regions and the Ice Age periods are well documented, almost no data are available for the

hot, arid regions such as Southeast Africa, the cradle of mankind or from Southwest Asia, where neolithisation took place. Does that really mean that no DNA has been preserved in bones from these regions or is it because the standard extraction and analytical methods that are relatively successful with bones from cold climates are inadequate for this more delicate material? I will discuss hereafter the results of our palaeogenetic analysis of the domestication of cattle, the problems that this kind of analysis raise, as well as the methodological solutions that enabled us to obtain results from hot, arid regions.

2. Domestication

2.1. Generalities of the domestication process

Domestication of animals and plants was a major step in the cultural evolution of man, the development, and spread of agro-pastoralism, and the foundation of modern farming societies. This process was coined the ‘Neolithic Revolution’ [24], since its importance is comparable to that of the Industrial Revolution. Agriculture and breeding of livestock enabled humans to produce food at a constant level necessary to feed a growing population [7,9,11,87]. The definition of domestication comprises a whole range of gradual differences. At one end of the spectrum, domestication can be considered as a form of behavioural co-evolution or even symbiosis [26,72,98]. At the other end, domestication is viewed as the control of animal populations by humans for a service or for use as primary material [47,101]. Domestication is presumably a gradual and fluid process with a longish transitory period of coexistence between two species before selective breeding is established, the consequence of which are complex shifts in population levels, technology, and social conditions. This cumulative process is characterised by increasing mutual dependence between, on the one hand, human populations and, on the other one, the animal and plant populations they target, with selective advantages for both partners [105].

2.2. The case of the domestication of ungulates in general and of the aurochs in particular

The first domestication of ungulates can be explained as a natural process resulting from a unique combination of environmental, biological, and social conditions that were prevalent simultaneously during a short geological time span in a very restricted area of the world, i.e., in Southwest Asia at the beginning of the present interglacial period [98]. Here the wild ancestors of all four primary meat-producing herbivores, i.e., bezoar (*Capra*

aegagrus), mouflon (*Ovis orientalis*), wild boar (*Sus scrofa*), and aurochs (*Bos primigenius*) were present during Neolithic times [96]. Unlike *Capra* and *Ovis*, however, *Bos primigenius* (the wild ancestor of present-day cattle) was present during the Pleistocene not only in Southwest Asia, but also in Europe and in North Africa. Therefore, it cannot be ruled out that the aurochs was domesticated not only in Southwest Asia, but also in Europe.

One potential area of cattle domestication is the Northern Fertile Crescent. Indeed, 8800–8300-year-old *Bos* remains that were smaller than average were to be found in the Upper Euphrates Valley, in Syria [48]. Size reduction, especially of males, thus revealing a reduction in the extent of sexual dimorphism, is a hallmark of cattle domestication [48]. The complexity of the domestication process, the incompleteness in time and space of the archaeozoological record, and the methodological limits all make it difficult, however, to pinpoint how and when the transition from hunting to husbandry took place [77]. Indeed, for the identification of forms of animal exploitation, archaeozoology relies on evidence such as biometrics, species frequencies, and demographic profiles in the archaeological record, which is subject to interpretation biases [25,47,69]. Some of these biases, such as bone fragmentation (particularly strong in cattle bones, [97]), partial burning, sexual dimorphism and morphological polymorphisms, can be circumvented by palaeogenetic analyses that are insensitive to these palaeontological biases. Both the archaeozoological and the palaeogenetic approach, however, face problems caused by the fact that the bone assemblages found in archaeological sites rarely reflect the composition and diversity of the natural populations. It is also difficult to discriminate between domesticates and their wild progenitors during the transitory period before breeding and husbandry began to leave morphological and genetic traces. The main morphological criterion for distinguishing between wild and domesticates is size. Indeed, it has been shown that ungulates tend to react with a reduction in body size to the changes imposed on them during the process of domestication, so that a significant reduction in body size may relate to (un)conscious selection in animal populations kept in captivity [95]. Size reduction, however, can also result from climatic deterioration, overhunting, or selective hunting of females [48]. Although climate affects the body size of herbivores, environmental conditions seem to have been fairly constant in the Upper Euphrates and Tigris Basin throughout much of the 9th and 8th millennia cal. BC. Its influence was also estimated by comparing bone measurements from consecutive populations of the gazelle (*Gazella subgutturosa*),

a species that has never been domesticated and whose body size is likely to have been determined by climatic phenomena only. Gazelle populations in this region did not show any significant fluctuation in size, ruling out that climate was responsible for the observed size reduction of the *Bos* bones [77]. To exclude selective hunting of females as the reason for this size reduction in the bone assemblages, their sex needs to be determined accurately [48]. *B. primigenius* was characterised by a striking sexual dimorphism with the result that female aurochs were no bigger, or sometimes even smaller than domestic bulls. This leads to an overlapping of the size distribution curves of the two species, the overlapping areas representing a very low confidence interval for the determination of individual bones [102]. Often, only the biggest bones, probably those of male aurochs, can be determined unambiguously as aurochs [99]. Therefore, the palaeontological discrimination of aurochs and cattle remains, at least in the areas and for the periods in which initial domestication took place, can be very misleading, and requires independent analyses that use different, complementary approaches to analyse certain aspects of domestication and population migrations [37]. One important approach is the study of the genetic signatures left by the domestication processes in the genomes of animals and plants (for an overview on goat, sheep, cattle, pigs, see [23,64]), especially since many changes do not directly affect the skeletons, but rather behaviour, development and maturation, which are unavailable to palaeontological analysis [4].

2.3. Genetic consequences of the domestication process

Animals are domesticated through continued breeding in captivity of animal populations that have originally been taken from the wild [100]. Thus, humans intervene in the life cycles of these animals, which ultimately enables these latter to increase in number and to extend the range of their habitats, even if humans might temporarily subject them to the impoverished conditions associated with initial human herd management [105]. Hence, humans increase the genetic fitness of the target animal populations and induce morphological, behavioural, and physiological changes [4,55,60,85,94]. The rates at which these changes appear depend on the conditions in which an animal was kept and the form of husbandry used to exploit it [4]. They are estimated from experimental and prehistoric data to occur within roughly 30 generations [14,15]. Large reductions in brain weight characteristic of highly domesticated animals such as dogs, sheep, and pigs, may take as

many as 100–200 generations [54]. By analysing genetic markers in the genomes of the domesticated animal populations and comparing them to those of the wild progenitors, both past and present-day genetic responses to their domestication can be found. These concern either neutrally evolving, non-coding DNA sequences, such as the hypervariable region of mitochondrial DNA, which reflect separation processes and migrations, or nuclear genes coding for phenotypic traits that were actively selected by man, thus causing modifications of allele distribution within the population [23].

The study of the domestication process via genetic analyses can be performed at two different levels: (i) the genomes of extant populations that have recorded the pre- and domestication processes, even though the domestication signal can be erased or masked by post-domestication processes; (ii) the genomes at the time of domestication, which reflect the situation and events that took place at that time. The state of chemical preservation of these ancient genomes normally only allows for the analysis of small samples and not for that of the vast populations that would be necessary to take fully into account the complexity of the domestication process.

2.3.1. Study of the genetic consequences of domestication as recorded in the mitochondrial DNA of modern populations

Population studies have been performed analysing the hypervariable region of mitochondrial DNA, which accumulates nucleotide substitutions more rapidly than nuclear DNA because it lacks many of the DNA repair systems that operate in nuclear genes and because it is not under strong selective pressure. It is therefore the ideal marker to study the divergence between wild and domestic populations under the relatively short timescale of domestication. Moreover, since its mode of transmission is maternal, it does not undergo recombination and is therefore the perfect target to trace back maternal lineages and population migrations. When using a molecular phylogenetic approach, however, it is sometimes impossible to date these evolutionary and migration events with precision, since the approach is based on the assumption that the molecular clock is linear and universal [31], which does not always hold true (for reviews, see [33,79,84]).

Analyses of the hypervariable region of mitochondrial DNA in modern populations of domesticated animals showed patterns that seemed to indicate the centres of domestication of the wild ancestors and subsequent migration routes of the domesticates [64]. For example, the data from a recent phylogenetic study of the mitochondrial hypervariable region in cattle from Southwest Asia, Africa and Europe showed a higher

mitochondrial diversity in the former region and a dramatic reduction in this diversity in Europe and Africa [93]. Curiously, the diversity in Great Britain, i.e., at the endpoint of the migrations waves in which cattle are supposed to have spread from Southwest Asia into Europe, seems to be higher than in the core region of Europe. It is difficult to explain how the diversity in the periphery of the migration area can be higher than in the core region if one does not assume that the reduction in genetic diversity post-dated the dispersal of the species. The data from phylogenetic studies on extant cattle cannot be taken as conclusive proof that Southwest Asia was the centre of domestication during the Neolithic for several reasons. Firstly, if the data are to reflect the actual situation, studies of genetic diversity require a stringent and well thought outsampling protocol, which is difficult to follow rigorously. Secondly, calculations of the rates of sequence evolution depend on calibration of the date of bifurcation between the outgroup and the wild ancestor, a calibration that relies on the fossil record (e.g., in the case of cattle [20,65]). As pointed out by Dobney and Larson [27], however, these dates are not point estimates, but instead are ranges, thus widening the confidence interval of the mutation rate estimates. Furthermore, they depend on the time depth of relationships within the data set, where closely related species will yield faster mutation rates than distantly related species [51]. This will lead to an overestimate of the date of splits within species, a serious problem when dating recent events such as domestication [50]. Thirdly, these data may not reveal the timing and succession of the events that occurred between the time of domestication, i.e., roughly 10,000 years ago, and the present-day situation. In particular, the present-day population structure might result not only from the initial domestication and subsequent migration events, but also from breeding and selection procedures during historical and even very recent times [13,66]. Epidemic diseases such as the bovine pest that ravaged the European cattle populations from the 6th to the 18th century [43] might also have caused a dramatic reduction in genetic diversity. Finally, with these data, it is not possible to determine the time at which the loss of diversity occurred, and they are therefore not suitable for the identification of either the centre of domestication of the aurochs or the subsequent events leading to this dramatic loss of mitochondrial DNA diversity in Europe and Africa. To establish the nature of these events, it is necessary to complement both approaches – the archaeozoological approach and the molecular phylogenetic one – with palaeogenetic studies, which allow for internal calibration of the domestication process. The analysis of the mitochondrial hypervariable region of DNA pre-

served in Neolithic aurochs and cattle remains from the presumed centre of domestication, Southwest Asia, and all along the Neolithic migration routes into Europe, the continental route over the Balkans and along the Danube [12] as well as the Mediterranean route [103,106], and the areas where these migration currents finally mingled, in France [57], should shed light on the domestication and dispersal processes of cattle.

2.4. Complementation of modern phylogenetic data by palaeogenetic data: the case of the domestication of the aurochs

Troy et al. [93] succeeded in retrieving mitochondrial DNA sequences from six Pleistocene aurochs roughly 12,300 to 3700 years old, which had roamed Great Britain at a time when it was not yet an island, but still connected to the continent, because the sea level was much lower. Their analysis, complemented by recent analyses of 42 more aurochs sequences from Mainland Europe [29], showed that these aurochs were phylogenetically too distant from present-day cattle to belong to the aurochs population that had been domesticated. In contrast, DNA sequences retrieved from the remains of five Mesolithic aurochs preserved in Italy, aged between 17,000 and 7000 years, were surprising, because they corresponded to the major European present-day mitochondrial haplogroup and, in three out of five cases, to the European consensus sequence [8]. This result suggests a large genetic diversity of the European *B. primigenius* populations and the possible domestication in the Adriatic area of local aurochs. Such an overrepresentation of the European consensus sequence, reflecting the present-day distribution of mitochondrial haplotypes amongst cattle from continental Europe, was also found in the vast majority of mitochondrial DNA sequences from Spanish and northern European Neolithic and Bronze Age cattle remains, even though for some of these remains dating and/or taxonomic attribution are uncertain [3,17,29]. The authors of the latter studies interpreted their data as an indirect confirmation of the hypothesis based on archaeological evidence that cattle were domesticated in Southwest Asia. Since contamination with modern European bovine DNA (see below) could produce an identical sequence distribution, it is important to complement these analyses with direct molecular evidence from Neolithic bovine remains from Southwest Asia.

When attempts were made to analyse DNA preserved in bone samples from Southwest Asia, they were not successful [30]. This was attributed to the hot, arid conditions ruling in large parts of this geographical region, since modelling has shown temperature to be one of

the major factors influencing DNA preservation [91]. Increased thermal fluctuation in bones was observed in the course of the excavation process [16]. DNA depurination, the most important DNA degradation route [22,58], is not affected, however, by thermal fluctuations between 4° and 70 °C, but rather by the average temperature [59]. Based on classical chemical thermodynamic equations (Arrhenius and first-order decay), one can calculate that a 10 °C increase in the average temperature (from 20° to 30 °C) for one year would cause the loss of only 3% of 100-base-pair-long DNA fragments, if the DNA in the fossils had the reactivity of DNA in solution (e.g., [82]). Therefore, temperature increase during excavation is unlikely to be responsible for significant DNA loss. The high average temperature, however, in most of the areas in which agriculture and domestication arose 10,000 years ago, i.e., Southwest Asia, may well be the primary cause of the poor preservation of DNA in bone remains. Indeed, one can predict that only one out of 10¹¹ copies of 100-base-pair-long DNA fragments would survive after 5000 years at 20 °C (as modelled in [82]). If DNA is so poorly preserved, only mitochondrial DNA sequences are expected at best to yield PCR amplification, since they are about two to three orders of magnitude more abundant than single-copy sequence in nuclear DNA [63,90]. This poor preservation renders ancient DNA analyses of these fossil remains very difficult, dooming them to both a high failure rate and false-positive results. What is needed are the development and establishment of rigorous experimental conditions that increase the sensitivity and the reliability of the palaeogenetic approach, and allow for the analysis of a critical number of bone remains from this area.

3. Palaeogenetic analysis of the genetic signature in fossil bone samples preserved in hot, arid climates: problems and solutions

3.1. Amplification of ancient DNA by the polymerase chain reaction

The smaller the quantity of preserved DNA is, the more difficult the retrieval of authentic genetic information will be. This is due to the fact that the analysis of tiny quantities of DNA requires the use of powerful multiplication techniques to obtain enough copies to be able to analyse them with the common methods of molecular biology. An enzymatic amplification process, called the polymerase chain reaction (PCR), has made it possible to multiply a single to a few DNA molecules and to end up with billions of copies of this target

molecule [70,86]. It was the power of this simple but ingenious method that revolutionised molecular biology and led to the blooming of the field of palaeogenetics (for recent reviews, see [76] and Hofreiter in this issue). PCR is performed by successive, reiterated cycles where the template DNA is first heat-denatured and then copied by a heat-resistant DNA polymerase, allowing the billion-fold multiplication of one or two DNA molecules initially present in the reaction mixture [70]. Since DNA in fossilising tissue is invariably heavily degraded, leading to its scarcity, this procedure is a choice method for ancient DNA research. The simplicity of its principle, however, hides its chemical complexity, and this lures users into an underestimation of the potential sources of error. This is particularly true for ancient DNA research. Here, the investigator has to contend with not only extremely low quantities and a high level of fragmentation of the preserved DNA molecules, but also DNA polymerase inhibitors, as yet chemically uncharacterised, which influence the synthesis reaction. Other problems include ions that change the fidelity of the DNA polymerase, and chemically modified nucleotide bases that have undergone post-mortem chemical reactions in the absence of repair, leading to reading and incorporation errors of the DNA polymerase [39,92]. Yet another threat to the reliability of the PCR process during the amplification of ancient DNA is jumping PCR. This is induced by nucleotide-base damage and leads to the formation of chimeric molecules [1,18,75].

Therefore, it is of utmost importance to control the PCR. This is possible when using a quantitative approach, and, in particular, quantitative real-time PCR (QPCR), which allows for *online* monitoring of the PCR and quantification of the amplified DNA thanks to the use of fluorescent probes [49]. In QPCR, the quantity of a given target molecule is compared to reference samples of previously determined concentration. The parameters that have to be optimised are specificity, efficiency, and reproducibility of the reactions. The format that is the most versatile, in that it permits monitoring of all events that take place during PCR, is the one that uses, as a fluorescent label, SyBr Green I, which interacts with double-stranded DNA, irrespective of its sequence. QPCR is much more stringent than conventional PCR, requiring the use of a ‘hot-start’ system [62]. With SyBr Green I, it is possible to apprehend the nature of the amplified fragments due to a terminal progressive denaturation stage, resulting in so-called ‘fusion curves’, the inflexion point (the maximum derivative) of which is considered the melting temperature T_M of the amplified molecules. In QPCR, the measurement of the T_M of the amplified fragments is similar to the gel

electrophoresis-based measurement of the size of the amplified molecules in ‘traditional PCR’ [62]. Therefore, it is possible to distinguish between the desired amplification product and the undesired primer–dimers without having to open the product-containing reaction vessels, thus minimising the risks of contamination of the laboratory environment with PCR products, an important consideration when working with ancient DNA [80]. QPCR is, moreover, particularly useful when optimising the PCR. Optimisation of the PCR for each primer pair is essential in ancient DNA research, where the PCR system is particularly challenged due to the low quantities of target molecules, their damaged nature, and the presence of inhibitors.

Therefore, QPCR is, to my mind, the best method for amplification of ancient DNA. Up until now, however, it has rarely been used and not a single study of the genetics of ancient populations or of a domestication process has been conducted using this method. We have adapted QPCR to ancient DNA research and used it for our study of the domestication process of the aurochs in Southwest Asia with a so far unequalled success rate. We have thus been able to appreciate better:

- DNA conservation, during and after burial;
- contamination with modern DNA;
- base modifications and amplification errors.

The method has proved to be a key asset in the study of DNA preserved in fossilising bones that have been subjected to climatic and/or chemical conditions that are unfavourable to DNA preservation. This will be discussed hereafter.

3.2. DNA preservation

3.2.1. Bone diagenesis

After death, bones do not always undergo total degradation but rather diagenesis, which transforms the initial inorganic and organic molecules and replaces most of the organic molecules by inorganic ones (fossilisation). During this process, the vast majority of DNA from the skeletal cells will be degraded. Under particular taphonomic (biological, physical, and chemical) conditions, however, DNA can escape total degradation and ‘survive’ for a long time. It is neither as yet known which factors (apart from temperature [91] and time), taphonomic agents and mechanisms are responsible for DNA preservation, nor whether there is an absolute time limit for DNA survival. Preservation of DNA in bones undergoing diagenesis is far from common (apart from that of permafrost samples), but is rather a lucky accident where

several taphonomic factors and agents unite. This is the case in permafrost conditions where DNA is preserved at a higher frequency and quantity than under temperate conditions. The ancient DNA molecules are invariably heavily fragmented, chemically modified, and complexed with other molecules [34,46,74,92]. The escape from total degradation of these small DNA fragments is suspected to be favoured in particular microenvironments within the fossilising bone, so-called molecular preservation niches [34], where DNA is protected from enzymatic degradation either via adsorption to apatite within interconnected crystals [88] or in colloids via complexation with humic substances and minerals, rendering it unavailable for purification and enzymatic amplification [34,36,38]. Such a preservation process implies a heterogeneous distribution of preserved DNA within the fossilising bone [35], which is indeed observed experimentally.

3.2.2. *Post-excavation DNA degradation*

When comparing the quantity of DNA preserved in fossil bones that had been subjected to normal archaeological procedures, i.e., washing, drying and storage in rooms without air conditioning, to the quantities preserved in freshly excavated, untouched bone material, we have shown by analysing some 250 fossil bones from various burial contexts in zones ranging from temperate to arid (including Southwest Asia) that DNA is much more quickly degraded after excavation than during burial [82]. In fact, the success rate of PCR amplification from freshly excavated fossil bones was more than twice as high (46%) than that from fossils excavated using standard procedures (18%). Moreover, the average quantity of about 150-base-pair-long DNA fragments was roughly six times higher in freshly excavated bones than in 'standard' ones. Furthermore, fossils preserved in hot, arid regions may contain so little retrievable, endogenous DNA that it is degraded totally and quite quickly when the fossils are treated with standard excavation and post-excavation procedures, whereas genetic information can still be retrieved when they are analysed in their 'dirt' state directly after excavation [82]. Modelling of the quantitative data obtained showed that the post-excavation degradation rate of DNA in fossils preserved in moderate climatic regions, such as those in northern France, could be around 70 times faster than in the sediment. The degradation is likely due to depurination of the DNA, a chemical reaction following a first-order kinetic [59]. This post-excavation decay of 90% of the endogenous DNA molecules can be caused simply by an average storage temperature that is higher than the preservation temperature, combined with a decrease in the

pH and a desalting effect due to the washing of the bone [82].

3.3. *Contamination with modern DNA*

3.3.1. *Post-excavation contamination of fossil remains*

The consequence of this fast degradation of endogenous DNA once fossil bones are unearthed, washed, dried and stored is that they are more likely to produce false-positive results than bones preserved in permafrost or cave environments. In fact, the less authentic endogenous DNA a bone contains, the higher the risk that contaminating modern DNA from the environment will be analysed. Recent studies traced the pre-laboratory contamination of Neolithic human teeth to the DNA from the excavators, who had contaminated the samples during excavation, washing and subsequent anthropological and genetic studies [89]. This type of direct contamination of bones was also observed when 200- and 3300-year-old human bones were deliberately mishandled [18]. Further studies confirmed and extended this result to ancient dog specimens preserved in museums that all were found contaminated with human DNA [67]. The contamination process involves water that originates from the sweat of the excavators and experimenters [18] and/or from the standard washing procedures of fossil bones after excavation [89]. Contradicting results were obtained concerning the depth to which the contaminating DNA can penetrate in the bone: while one study showed that contamination occurs only on the outer 1–2 mm of the bones [18], another study demonstrated that handling of medieval human bones that had been excavated in the 1970s did not contaminate the bones with the experimenter's DNA [41]. Only DNA that was not specific to the experimenters was found and this was interpreted as stemming from the initial excavators and curators. An explanation for this observation is that buried fossilising bones and teeth might be very susceptible to contamination with environmental modern DNA during their excavation, when the pores of the calcified tissue are still open, whereas these collapse during post-excavation desiccation [41]. The contaminating exogenous DNA can show the same nucleotide base substitutions as ancient DNA due to subsequent base damage, and can therefore mimic ancient DNA, leading to erroneous results [89]. In contrast, other authors have claimed that contaminating DNA could be preferentially destroyed by bleach treatment of the bone powder [68]. In fact, these authors claim that contaminating DNA may be distinguished from authentic DNA based on the relative proportions of short and long frag-

ments that can be amplified. This demonstration was not fully convincing, however, since the ratio between the quantities of supposedly authentic short to longer DNA fragments was not related to their absolute quantity, a correlation that would be expected if the interpretation were correct. The observed effect might rather have been caused by a combination of phenomena involving PCR inhibition, loss of DNA through bleach treatment, differences in the amplification efficiencies of the various primer sets, and/or carry-over or reagent contamination with modern DNA (see below).

To conclude, contamination of bones with environmental DNA occurs during handling, but may largely be prevented when bones are excavated and analysed with gloves and when washing is avoided.

3.3.2. Contamination of extraction chemicals and PCR reagents

The downside of the powerful method of PCR is its sensitivity to contamination with modern DNA molecules that are preferentially amplified. This is particularly problematic in the study of human remains, but also when domesticated animals, such as cattle and pigs, are studied because of the omnipresence of their DNA in our environment. These molecules are present in every laboratory, conveyed by the experimenters, and also present in the reagents, in particular, in deoxynucleotide preparations made from hydrolysed cattle or pig products [56], as well as in commonly used enzyme-stabilising proteins such as bovine serum albumin or gelatine (Champlot et al., in preparation). These modern contaminating molecules can easily be misinterpreted as authentic endogenous ancient DNA molecules in fossil samples where no or very little endogenous ancient DNA is preserved. This can lead to erroneous results reflecting the use of products of certain extant animal breeds for the production of reagents or the presence of certain human lineages amongst the employees of biotechnology companies involved in the production of the reagents, rather than the true past genetic diversity of aurochs, cattle, wild boars, pigs, and humans. For these reasons, not only the excavation and storage methods, but also the analytical ones have to be carefully monitored when working with fossil samples, in particular with human, bovine, and porcine samples that had been preserved in geographical areas and burial contexts in which the climatic and taphonomic conditions are unfavourable to DNA preservation. QPCR has proved to be the best method for careful quantification of both contamination by reagents and quantity of DNA found in fossil extracts. For our study of cattle domestication, we conducted a systematic quantitative analysis of reagent contamina-

tion to ensure the reliability of the results we obtained. We gamma-irradiated plastic ware and reaction water, and prepared large batches of reaction mixes. For each reaction mix, we established the frequency with which a mock positive amplification was observed and the quantities of molecules involved (we did not validate mixes that contained more than one molecule per ten reactions). We systematically sequenced a large number of mitochondrial DNA contaminants found in various batches of reagents over the years and observed that the contaminating sequences corresponded almost exclusively to the dominant European consensus sequence. Amplification from fossil extracts was considered reliable only when the frequency of occurrence of positive PCRs out of various amplifications was well above that seen with reagents alone. Such controls are critically required, especially when a sequence corresponding to the major European haplotype is obtained. Unfortunately, such rigorous monitoring was sadly lacking in a number of studies that reported bovine sequences identical to the major present-day haplotype, and hence the value of much of the literature reporting palaeogenetic studies of bovine (and porcine) domestication is limited.

3.3.3. Carry-over contamination

Quantitative high-fidelity PCR systems help to increase the reliability of the production of DNA sequences from fossil extracts. This is a critical issue even for palaeogenomics [42,53,71,78], where the results obtained by large-scale sequencing need to be confirmed by PCR (see Hofreiter, this issue). When QPCR is routinely coupled with enzymatic fragmentation of DNA molecules produced in previous PCR and cloning steps, this can overcome one of the most severe threats to the authenticity of ancient DNA sequences, namely carry-over contamination. Since each PCR and each bacterial colony after cloning produces up to a billion copies of the original target molecule, concentrated in one tube, the danger of spreading these molecules is enormous. This is a major concern, since these molecules are completely identical to the original target molecule. It can distort in-house reproduction of previously retrieved data. Physical containment, positive air-pressure, UV-irradiation, and one-way circulation practices in the laboratory cannot be expected to decrease this contamination source to zero. We are routinely using a QPCR procedure with dUTP during PCR amplification, and preincubate each PCR with UNG to degrade potential contaminants from previous reactions. We have established that this UNG-coupled quantitative real-time PCR (UQPCR) eliminates 99.99% of DNA molecules from previous PCRs [81]. We also perform our cloning steps

in an *E. coli* strain that incorporates dUTP in DNA, which allows us to degrade 94% of potential contaminant plasmid molecules [81].

By using UQPCR in conjunction with strict physical containment procedures, we add a considerable level of reliability to our analyses that cannot be attained with the strictest physical containment procedures alone, because no procedure can eradicate a major vector of contamination: the experimenter (see § 2.3.1).

3.4. PCR errors and variability

QPCR for the analysis of ancient DNA can afford detailed insight into the kinetics of the PCR at two levels at least. It allows (i) quantification of the target molecules in the fossil extract and (ii) quantification of the inhibitory effect of the extract [80]. The quantification of the target molecules and primer–dimers that are potentially formed allows for optimisation of the PCR conditions, thus ensuring an increased sensitivity and fidelity for the amplification of a very small number of target molecules. This is possible only via a stringent quantitative approach including the testing of at least as many negative controls as samples. Indeed, sporadic low-level contamination with modern DNA molecules can be masked by the production of primer–dimers if the dimers are not correctly detected and their synthesis threshold quantitatively measured. Since the amplification of primer–dimers is very efficient once the first dimers have been elongated, they can be amplified faster than the *bona fide* product and thus use up all primers, preventing amplification to a detectable level of the correct product or contaminant [10]. Primer–dimers, however, occur stochastically and they are not detected regularly at a specific cycle number, but they rather appear within a certain cycle number range differing between different primer pairs. If this range encompasses the cycle number at which contaminating molecules can be detected, contamination will be sporadically detected only when primer–dimers are produced at a late stage of the PCR. In this case, contaminating molecules, even if relatively abundant, might be detected as rarely as ‘fossil’ DNA. It is thus essential to detect correctly primer–dimers, which often remain undetected on standard agarose gels, and to determine the time of appearance, i.e., whether they appear before or after the targeted PCR products corresponding to a single initial molecule. Moreover, fossil extracts contain substances of as yet unknown chemical identity that inhibit the DNA polymerase Taq or affect primer–dimer formation, thus requiring the quantification of amplifiable material and of the PCR performance for each fossil extract.

3.4.1. Increasing PCR fidelity

When the two procedures, QPCR and UNG treatment, are combined in the so-called UQPCR [81], not only carry-over contamination is prevented, but also the error rate of nucleotide incorporation during PCR is considerably reduced. This is attributed to two phenomena. First, the enzyme uracil-*N*-glycosylase (UNG) eliminates the majority of post-mortem nucleotide-base lesions occurring in ancient DNA, namely deaminated cytosines [52,92], thus preventing the major causes of erroneous copies of the original template. Second, we have established a strategy that most likely increases PCR fidelity. Indeed, fossil extracts contain not only inhibitors that decrease PCR efficiency, but also compounds that affect the fidelity of the Taq DNA polymerase, such as the ions Mn^{2+} [28], which are very often concentrated in fossilising bones and possibly co-purified bound to humic substances. We systematically quantify the inhibitory effect of each fossil extract on a dilution series of an internal DNA template and precisely dilute the extracts to minimise inhibition [80]. We believe that this also permits the dilution of the inhibitors that affect the fidelity of the reaction. Indeed, the percentage of non-synonymous base substitutions that we detected after sequencing of the resulting UQPCR products and clones amount to only 0.024% type-2 transitions and 0.046% type-1 transitions [83], whereas standard procedures report higher values: 0.18% and 0.11%, respectively [40], or 0.25 to 0.8% [88]. Therefore, this procedure considerably increases the fidelity of the amplification of ancient DNA molecules, yielding sequences that are more reliable.

An increase in PCR fidelity was also achieved via treatment of fossil extracts with hypochlorite [88]. Although this treatment was apparently relatively efficient in terms of fidelity, it was costly in terms of successful PCR amplification, since it caused the loss of 99% of the ancient DNA molecules present in the fossil bone [88]. In contrast, UQPCR achieves an even lower level of non-synonymous substitutions in the PCR products [83], while causing the loss of only a few molecules in only a subset of the fossil extracts (Champlot et al., in preparation).

3.4.2. Establishment of the consensus sequence

In most palaeogenetic studies, the deduction of the authentic ancient DNA sequence is based on the evaluation of a certain number of clones. This number is crucial for the reliability of the deduced sequence. In 2005, Bower et al. [19] claimed that a minimum of 12 clones is necessary to deduce a correct consensus sequence from a fossil sample. This minimum number can rise to 30 clones per PCR product if the heterogeneity

of the sequences is higher than average or if jumping PCR phenomena occur generating recombinant PCR products [21,104]. Using an *in vitro* assay, we were able to show, however, that direct sequencing of the PCR product is as powerful for the deduction of the consensus sequence as the proposed sequencing of 20 clones, because the majority sequence is directly read, while a 20% contamination with another DNA sequence can be easily detected [83]. Using these procedures on at least four PCR products per fossil sample, i.e., two independent PCRs from two different extracts of each sample, a high degree of reliability for the production of sequences from fossil extracts is guaranteed, so far equalled only by the procedure described by Stiller et al. [92]. To exclude post-mortem damage and polymerase misincorporations, these authors validated a sequence only if two lots of 12 clones obtained from two independent PCRs were 100% identical. Unfortunately, this seems to be the only study conducted according to these strict but very necessary rules. Instead, when the methods used lead to a high nucleotide misincorporation rate, consensus sequences are often determined in a somewhat arbitrary fashion from small heterogeneous sequence data sets, in which the chosen ‘consensus’ can sometimes differ from the majority of the clones found within some PCR products (e.g., [73]), or, even worse, differ from the sequence obtained directly from the PCR product [32].

3.5. *QPCR*: Conclusion

We relied on the established quantitative PCR approach for the analysis of the cattle domestication process in Southwest Asia during the Neolithic. We obtained a set of solid data thanks to:

- quantification of the initial target DNA molecules in our PCR mixes, since the reliability of a PCR product is a function of the quantity of the template;
- elimination during PCR of the major post-mortem base damage;
- a low polymerase error rate possibly due to optimal dilution of the fossil extracts;
- quantification of reagent contamination;
- preferential analysis of freshly excavated fossil bone samples.

4. Palaeogenetic analysis of the cattle domestication processes: from the beginning to the end

We carried out a large-scale palaeogenetic analysis of Neolithic and Bronze Age *Bos* remains from Sou-

thwest Asia relying on the UQPCR procedure. This is the first palaeopopulation genetic study to be entirely performed by this quantitative, sensitive, and reliable approach. Moreover, for our study of cattle remains from regions known for their poor preservation of DNA, such as northeastern Syria, we used not only bone remains from collections, but also freshly excavated fossil bones. Indeed, amongst these samples from regions characterised by hot, arid climatic conditions or wide fluctuations in temperature (hot summers, cold winters), only the freshly excavated, unwashed and refrigerated bone samples yielded genetic results.

To elucidate the processes of domestication and the spread of cattle and the subsequent evolution of genetic diversity, and also to ascertain whether secondary domestication events took place in western Europe, we analysed roughly 230 fossil bone samples originating from around 65 archaeological sites in the putative centre of initial domestication, Southwest Asia, and in western Europe, where the two Neolithic migration waves met [2,57,103], in particular, the territory that constitutes modern-day France (Pruvost et al., in preparation). The combination of the use of freshly unearthed, unwashed fossil remains and our quantitative high-fidelity and high-sensitivity PCR approach allowed us to obtain some 60 authenticated mitochondrial sequences from between 9500- and 3000-year-old bovine remains, 30 from Southwest Asia and 30 from western Europe (mainly France) (Pruvost et al., in preparation). This number of sequences, relatively high for a palaeogenetic study, allowed us to carry out a statistical analysis of the obtained sequences based on a serial sampling coalescence simulation algorithm allowing for time spacing between subsets of sequences (F. Depaulis, pers. comm.). The use of population genetics makes it possible to compare groups of sequences and to evaluate the statistical significance of these comparisons, rather than to focus on single haplotypes, thus diminishing the effects of small sample size spread over periods of sometimes considerable length. The result of this large-scale analysis proves on the genetic level that it was the aurochs population from the Upper Euphrates and Tigris Basin that had been domesticated almost 10,000 years ago and then spread into Europe, since Neolithic and Bronze Age cattle in western Europe show a mitochondrial signature that is a subset of that of their ancestral populations in Southwest Asia (Pruvost et al., in preparation). They also gave rise to the extant cattle populations all over the world, although modern-day cattle in continental Europe represent only a small subset of the original populations (Pruvost et al., in preparation). In contrast, the western European aurochs did not leave a mitochondrial signature in extant

European cattle, which indicates that female western European aurochs were not domesticated in Europe, but rather maintained their status as hunted game until at least the Bronze Age (Pruvost et al., in preparation). This is consistent with the conclusions drawn from osteometrical data collected from a huge sample (100,000 fossil bones from the Neolithic and 130,000 fossil bones from the Bronze and Iron Ages) covering 5000 years from the Aisne valley in northern France [5,6,44,45]. We did obtain, however, molecular evidence for hybridisation between male aurochs and female domesticated cattle, occurring at low frequency in northern France during the Neolithic and the Bronze Age, producing viable offspring, through a molecular sexing experiment, which is based on the sizes of the amelogenin alleles that are different on the bovine X and Y chromosomes (Pruvost et al., in preparation). More data are needed to ascertain the frequency of this event, which one would expect to decrease with time as a function of the extinction of the wild aurochs in France during the 10th century [61]. These fertile offspring are likely to have been culled due to their lack of docility, and therefore did not leave a long-term genetic signature in the gene pool.

An interesting result of our study concerns the evolution of the genetic diversity from the Neolithic to the Present. In contrast to the West European aurochs populations that were found to be characterised by a low-level diversity, which did not significantly evolve during the last 10,000 years, the diversity of the bovine populations during the Neolithic and the Bronze Age was greater than that of modern cattle in Europe and Africa. This difference suggests that the present-day low genetic diversity of cattle, particularly in mainland Europe, is the result of events that postdate the Bronze Age period and occurred with all likelihood during historical times. This result probably shows the influence of the selection and breeding procedures and of epidemic diseases on the evolution of the gene pool of domesticated animals.

5. Conclusion

Methodological progress concerning both the excavation procedures in the field and the DNA amplification procedures via PCR allows retrieval of ancient DNA sequences from bone samples preserved in climate zones and burial contexts unfavourable to DNA preservation. Special excavation procedures prevent the post-excavation degradation of DNA, thus increasing the success rate of palaeogenetic analyses. Quantitative real-time PCR increases the sensitivity and fidelity of the amplification procedure, and therefore the reliability of DNA sequences produced from human and animal fos-

sil bones, and should become the standard procedure for any palaeogenetic research.

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