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Adaptor-mediated amplification of minute amounts of severely fragmented ancient nucleic acids

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Abstract. It has been repeatedly shown that high copy number mitochondrial DNA sequences can be recovered from ancient samples. A significant increase in the volume of information available to researchers will be observed when the amplification of nuclear DNA becomes commonplace and reproducible. To this end we established a modification of the Rapid Amplification of cDNA Ends (RACE) procedure normally used for the generation of cDNA ends from adaptor-ligated expressed sequence tag libraries. The modifications were designed to specifically address the problems associated with the highly damaged nucleic acids extracted from palaeontological specimens. For this study we used 6 human samples dating to 450 AD and ~6.500 BP that were refractory to reliable amplification of single copy loci by PCR. Racemate contents (ratio of D/L enantiomers) of aspartic acid, alanine, and leucine also indicated that no amplifiable DNA is present in 5 of the 6 samples. The proposed technique allowed us (i) to amplify four X-chromosomal loci from 5 human specimens, and (ii) to correct allelic drop-out phenomena at the amelogenin locus in one individual; thus showing that the threshold of 80 $\times 10^{-3}$ for D/L_{asp} as a borderline for the presence/absence of amplifiable aDNA requires reassessment. Reliability of the proposed technique (i.e. amplification of DNA sequences endogenous to the find) was validated by the application of "ancient RACE" (aRACE) to prehistoric animal samples.

Key words: adaptor ligation, ancient DNA, PCR, RACE, sex typing, short tandem repeats, template improvement.

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Introduction

Numerous obstructions to research are apparent when working with ancient hard or soft tissue samples. Generally, a low efficiency of PCR with ancient DNA (aDNA) as template is observed. Since most of the causative agents for this problem are only poorly understood, the field of palaeogenetics is still awaiting a thorough exploration (HERRMANN, HUMMEL 1994). The starting point is the assessment of DNA survival in an ancient specimen. POINAR and colleagues (1996) found that the ratio of D to L enantiomers of certain amino acids provide such an indication. Given the putative presence of aDNA, a number of advances have been made, which may increase amplification efficiencies: (i) the optimization of the aDNA extraction procedure to increase both the quality and quantity of nucleic acids endogenous to the find (PÄÄBO 1989, MEIJER et al. 1992, HÖSS, PÄÄBO 1993, PETRISHCHEV et al. 1993, POINAR 1994, TUROSS 1994, HÄNNI et al. 1995, CATTANEO et al. 1997, PUSCH, SCHOLZ 1997, SCHOLZ, PUSCH 1997, YANG et al. 1998); (ii) the removal of PCR inhibitors by selective purification steps (PÄÄBO et al. 1989, PÄÄBO 1989, HAGELBERG, CLEGG 1991, BROWN, BROWN 1992, GOODYEAR et al. 1994, TUROSS 1994, HÄNNI et al. 1995, SCHOLZ et al. 1998); (iii) the monitoring of contaminating DNA derived from microorganisms (PÄÄBO 1989, PUSCH, SCHOLZ 1997) or modern humans (SCHMIDT et al. 1995, STONEKING 1995, PUSCH et al. 2000a); (iv) the use of specially adapted PCR protocols (HAGELBERG, CLEGG 1991, THOMAS, PÄÄBO 1993, MCPHERSON et al. 1994, HÖSS et al. 1994, HANDT et al. 1994, PUSCH et al. 2000b) to permit the amplification of minute amounts of highly damaged target DNA; and (v) the modification of aDNA itself prior to amplification (FOO et al. 1992, HERRMANN, HUMMEL 1994, PUSCH et al. 1998, POINAR et al. 1998).

Whereas points (i)-(iv) have been widely addressed by numerous protocols, the last strategy (v) offers a great scope for innovation. In order to circumvent the restrictions of both low copy number and fragmented ancient nucleic acids, we modified the Rapid Amplification of cDNA Ends (RACE) procedure to substitute cDNA synthesis from mRNA by repair of aDNA molecules, which led to the term ancient RACE (aRACE).

Following creation of repaired duplex DNA (PUSCH et al. 1998) the ancient DNA fragments are ligated to DNA adaptors. This adaptor is partially double stranded and is phosphorylated at the 5′ end to facilitate blunt-end ligation of the adaptor to both ends of the duplex aDNA by T4 DNA ligase, resulting in molecules containing an identical terminal structure. Prior to aRACE, the adaptor-ligated DNA is diluted to a concentration suitable for semi-specific amplification. aRACE PCRs are primed with an internal sequence-specific primer (SP) and one of the adaptor primers (AP1, AP2). The adaptor-ligated aDNA does not contain a binding site for either the AP1 or AP2 primer. During the first round of thermal cycling, the inner SP primer is extended, creating AP1/2 binding sites at the 5′ (or 3′) terminus of the nucleic acids. In subsequent cycles, both AP1/2

and SP primers can bind, allowing an exponential and highly specific increase in the amount of the aDNA segment of interest. Thus, aRACE is used to exponentially increase the minute amounts of aDNA normally isolated from prehistoric samples.

The presence of severely fragmented duplex aDNA (D/L_{asp} of $> 120 \times 10^{-3}$) in a sample may restrict efficient PCR as two opposite (i.e. complementary-oriented) PCR primers are unable to amplify a contiguous DNA segment with a length greater than ~70 bp (including primer sites). This could be explained by the fact that (i) in short aDNA fragments one PCR primer site may exist, but the second and complementary primer site does not (i.e. no amplification); (ii) the target molecule is underrepresented in the pool of isolated aDNA fragments (i.e. PCR jumping phenomena); or (iii) degradation has left one or both primer sites altered (i.e. no amplification, PCR slippage or non-specific amplification). This is complemented by the use of an intact "joker" sequence (the unique adaptor) in aRACE. The use of adaptors permits the amplification of DNA segments from a sample in a semi-specific PCR (aRACE1). This greatly enhances the chance of amplifying the specific target in a subsequent nested (i.e. target-specific) PCR (aRACE2). aRACE may therefore be an essential prerequisite when single copy sequences of the nuclear genome are targeted. To date such segments have only been amplified serendipitously.

Material and methods

Specimens

The remains of five human individuals discovered during the 1996 archaeological excavations at the pre-pottery neolithic site (~6.000 BP) at al-Buhais 18 (United Arab Emirates) were used for a short tandem repeat (STR) typing on 4 genomic loci. Repository identification of the specimens is alBCS, alBCR, alBCU, alBAB and alBXB. Also, a merowingian individual dating to 450 AD, and excavated in Neresheim, Germany, was included in the analysis. Ancient control specimens were derived from pig (Heuneburg, Germany) and sheep (Grundfeld, Germany), dating to 2.700 BP and ~3.000 BP, respectively.

Precautions and contamination monitoring

Samples were removed from the distal or proximal ends of the diaphyses of long bones. In order to monitor and thus to minimize the presence of contaminating DNA, several controls and additional precautions were performed along with the experiments, and these are described in detail elsewhere (PUSCH, SCHOLZ 1997, SCHOLZ, PUSCH 1997, PUSCH et al. 1998, SCHOLZ et al. 1998). For analysis of degraded amino acids, the amino acid derivatives out of 1 mg of dried pulver-

ized *Compacta* were analysed by SIM-GCMS on a Chirasil-Val column, as described in HODGES, SMITH (1994).

DNA processing and amplification

For the extraction of highly degraded nucleic acids a combination of two slightly modified protocols was utilized (PUSCH, SCHOLZ 1997, SCHOLZ, PUSCH 1997). Briefly, precipitation of DNA was performed in the presence of 0.5 volume of 4 M ammonium acetate and 2.5 volume of room-temperature 95% ethanol, mixed and then centrifuged immediately in a microcentrifuge at 14,000 rpm for 30 min at room-temperature. The pellet was washed twice in 200 µl of 80% ethanol, and was dissolved in Tricine-EDTA buffer (10 mM Tricine-KOH, pH 8.5, 0.2 mM EDTA). In order to minimize inhibitory effects and the random priming of small aDNA fragments in subsequent amplification approaches, we excluded molecules < 70 bp by gel extraction via a protocol published by PUSCH (1997a). Repair of duplex aDNA was essentially carried out as described in PUSCH et al. (1998). For adaptor ligation and PCR-mediated amplification we followed the manufacturer's recommendations (Marathon™ cDNA amplification kit, Clontech). For the amplification of the X-chromosomal loci DXS1227 (FAM), DXS1055 (TET), DXS1106 (HEX), and DXS990 (FAM), the PCR assays were carried out according to the guidelines of the ABI PRISM linkage mapping set (Applied Biosystems-Perkin Elmer) using a PE 9600 thermal cycler. For the amplification of the X/Y-dimorphic locus amelogenin, a standard 50 µl PCR set-up was applied and temperature profiles were designed as a standard touch-down protocol using primers AmelA (5′-CCCTGGGCTCTGTAAAGAATAGTG-3′) and AmelB (5′-ATCAGAGCTTAAACTGGGAAGCTG-3′).

Identification of radioactively and dye-labelled alleles

Amelogenin-specific PCR products were separated on a 15% polyacrylamide gel running in 1.5 × TBE buffer. After treatment of the gel as described in PUSCH (1997b), the nucleic acids were subsequently transferred onto Qiabrane plus membranes (Qiagen) by capillary transfer in 0.025 M KOH and 1 M NaCl. The membrane was soaked in 2× SSC and pre-hybridized overnight in 8 ml of HybrisolXR™ (Oncor) at 45°C. Fifty µg/ml unlabelled sonified salmon sperm DNA, 50 µg/ml heparin and 10% dextran-sulfate were added. The oligonucleotide 5'-GTTTCTCAAGTGGTCCYRATTTTACAGTTC-3' was labelled within 20 min at 37°C using 1.5 MBq [γ^{32}P]-ATP (Amersham) and 1 U of polynucleotide kinase (Boehringer Mannheim). The probe was then purified by passage through a polymer cotton/sephadex G-50 column at room temperature. The hybridization was carried out at 45°C for 15 h. Following hybridization, the filter was washed twice for 20 min in 4 × SSC and 0.15% SDS at 50°C, and twice for 5 min in 0.1 × SSC, 0.1% SDS at 40°C. The filter was exposed to an X-ray film at –80°C for 8 h.

Dye-labelled X-chromosomal alleles were mixed in the ratio 1 FAM (blue): 2 TET (green): 4 HEX (yellow), and fractionated on a 373A automated sequencer using filter wheel set B. Alleles were identified using an internal length standard (red) and the GeneScan software (Perkin Elmer-Cetus).

Results

Characterization of amino acids

According to POINAR et al. (1996), the degree of racemization of amino acids can be used as an indicator for the probable extent of DNA survival in prehistoric material. This can also be used for a rough estimation of the length of PCR products obtainable from a sample (POINAR et al. 1996, KRINGS et al. 1997, COOPER et al. 1997). Briefly, the higher the value obtained for D/L_{asp}, the smaller the size of products that may be generated by PCR. We measured the extent of racemization of the indicative amino acids, i.e., aspartic acid (asp), alanine (ala) and leucine (leu), by the SIM-GCMS method, using deuterium exchange to differentiate between racemate originally present in the sample and racemate arising from the hydrolysis and work-up of the sample (HODGES, SMITH 1994).

Table 1. Racemate contents (D/L ratio) of bone samples

Individual	Specimen	Alanine	Leucine	Aspartic acid
alBXB	femur	206×10^{-3}	78×10^{-3}	419×10^{-3}
alBCR	femur	137×10^{-3}	47×10^{-3}	309×10^{-3}
alBCS	ulna	157×10^{-3}	68×10^{-3}	338×10^{-3}
alBCU	clavicula (2^{nd})	217×10^{-3}	109×10^{-3}	413×10^{-3}
alBAB	femur	162×10^{-3}	9×10^{-3}	422×10^{-3}
Neresheim	tibia	2×10^{-3}	2×10^{-3}	40×10^{-3}
Modern control	tibia	2×10^{-3}	1×10^{-3}	8×10^{-3}

Only one criterium proposed by POINAR et al. (1996) as a prerequisite for the presence of amplifiable DNA in ancient samples (D/L_{ala} and $D/L_{leu} < D/L_{asp}$) was fulfilled by all ancient samples measured (Table 1). This is an indicator for the non-contaminated status of the respective bone powder. The second criterion ($D/L_{asp} < 80/117 \times 10^{-3}$), however, was not fulfilled by the 5 samples from the Stone Age burial ground at al-Buhais, thus indicating that the DNA extracts should be refractory to PCR amplification (POINAR et al. 1996, KRINGS et al. 1997, COOPER et al. 1997). In contrast, the Neresheim specimen fulfilled the second criterion, thus indicating that amplifiable DNA is likely to be present within the sample.

Analysis of degraded nucleic acids

In order to prove the efficiency of aRACE, we tested the 1,550-year-old human individual from Neresheim (Germany) for sex. In previous test series, PCR-mediated amplification of the X/Y-dimorphic amelogenin locus was without success when standard techniques for the extraction and amplification of ancient DNA were utilized (e.g. HÖSS, PÄÄBO 1993, PUSCH, SCHOLZ 1997, SCHOLZ, PUSCH 1997). These amplifications repeatedly generated a sole 112 bp band (Figure 1), which appears to be an artificial PCR result; the co-amplification of a 106 bp product is evidence for male origin (106 bp-112 bp). In contrast, a female sexing depends on the homozygous constellation of X-chromosomal alleles (106 bp-106 bp). Thus, the previous sex typing of the Neresheim individual was erroneous due to the phenomenon known as "allelic drop-out" (the lack of a single allele of a "heterozygous" genotype). To obtain the authentic genotype we amplified the amelogenin locus with 4 primers using the adaptor-ligated DNA from 4 individual aRACE1 reactions (AmelA/SP1-AP1; AmelA/SP1-AP2; AmelB/SP2-AP1; AmelB/SP2-AP2). Whereas all aRACE2-specific PCRs utilizing the AP1 primer failed in subsequent AmelA/SP1-AmelB/SP2 PCRs, the aRACE2 PCRs generated by the primers AmelA/SP1-AP2 were successful. Unexpectedly, aRACE1 reactions using AmelB/SP2 and AP2 primers were refractory to amplification in the specific aRACE2 (AmelA/SP1-AmelB/SP2) reactions. Using an amelogenin-specific oligonucleotide as probe in Southern hybridization, the favourable effect of the exclusively repaired (but not adaptor-ligated) molecules is demonstrated in Figure 1; a faint band at position 106 bp (panel C, lane 2) is visible on the X-ray.

In summary, in three approaches using aRACE1-generated DNA (AmelA/SP1 and AP2 oligonucleotides) in AmelA/SP1-AmelB/SP2-specific PCRs, the dimorphism (i.e. male evidence) at the amelogenin locus was identified (Figure 1).

To further test the aRACE procedure, we amplified polymorphic STR sequences from five human individuals of the Stone Age burial site at al-Buhais. The samples had been analysed by SIM-GCMS, and had been found to harbour non-amplifiable nucleic acids (Table 1). In agreement with that presupposition, aDNA isolates from all five individuals gave no or inconsistent results when standard amplification techniques were applied (SCHOLZ, PUSCH 2000). We therefore used aRACE DNA of the al-Buhais specimens for a new test series with polymorphic X-chromosomal markers covering an amplicon size range of 76 bp-130 bp. Since we determined the AP2 primer to be the better starting point for amplification from the adaptor sites of the modified aDNA library, we chose to perform aRACE1 reactions with AP2 and either the forward (A or SP1) or the reverse (B or SP2) primer out of the ABI PRISM linkage mapping set. Following minor PCR optimization strategies, all DNA markers were informative in the individuals tested. STR DXS990 with the largest allele sizes of 122-130 bp, however was refractory to amplification in the initial test series. Typing of the al-Buhais individuals using DXS990 was completed after two re-amplifications of 1 µl of 1/200 dilutions of the respective first round aRACE2 reactions. Success of the finger-

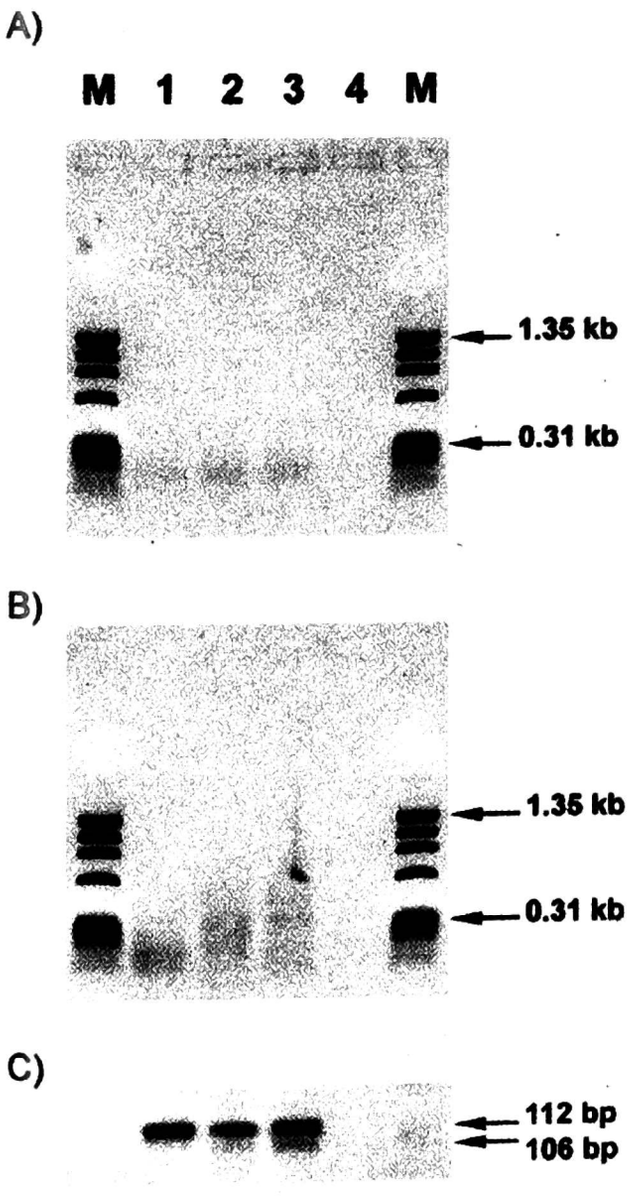
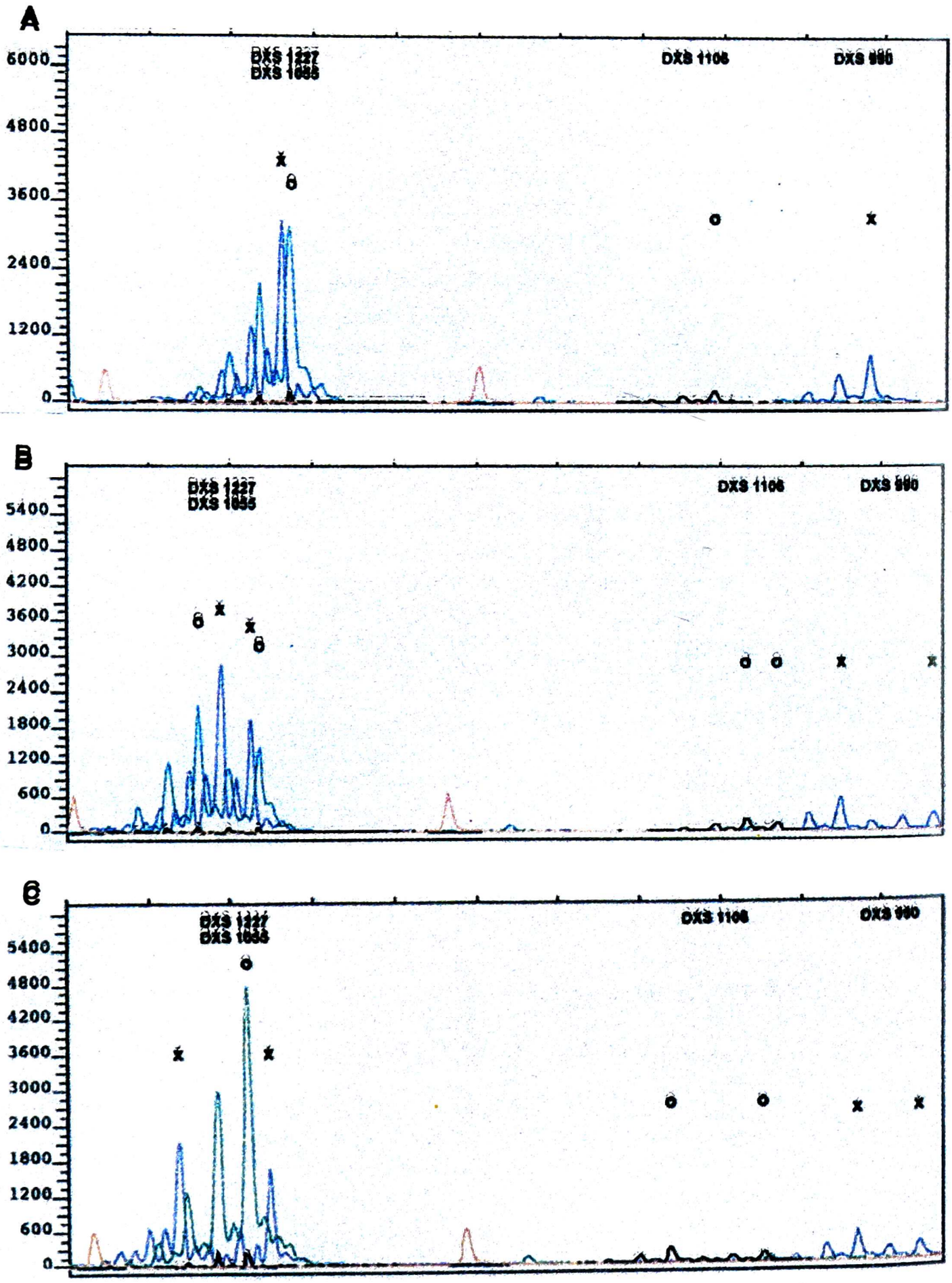


Figure 1. Comparison of differently processed aDNA extracts. Note that all isolates as well as amplicons had been gel-extracted in order to exclude molecules <50-70 bp from further analyses. Lanes 1, test series using native (i.e. unmodified) aDNA; lanes 2, RR-improved aDNA; lanes 3, RR-treated DNA plus ligation of adaptors; lanes 4, blank extraction control using no DNA. Corresponding unspecific multiplication of the dimorphic locus amelogenin (lanes 1, 2 and 4, pooled primer extension products; lane 3, aRACE1, i.e. semi-specific PCR) are shown in panel B. Subsequent specific amplification via PCR (lanes 1, 2, and 4) and the aRACE2 approach (lane 3) is visualized in panel C.

Template for amelogenin-based amplifications was derived from the Neresheim individual, Germany. DNA was separated on either agarose gel (panels A and B) or on polyacrylamide gel (panel C). Gels were subsequently stained with ethidium bromide, surveyed under 254 nm UV transillumination and photographed (EASY, Herolab). In panels A and B a negative image was captured. Panel C is the autoradiographic image of a Southern hybridization using an amelogenin-specific 30mer oligonucleotide as the probe. Sizes are given in kilobase pairs (panels A and B) or base pairs (panel C). Lanes M show DNA standard marker ΦX174/*Hae*III in panels A and B, and CD4 DNA standard (Serac) in panel C.


A
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B
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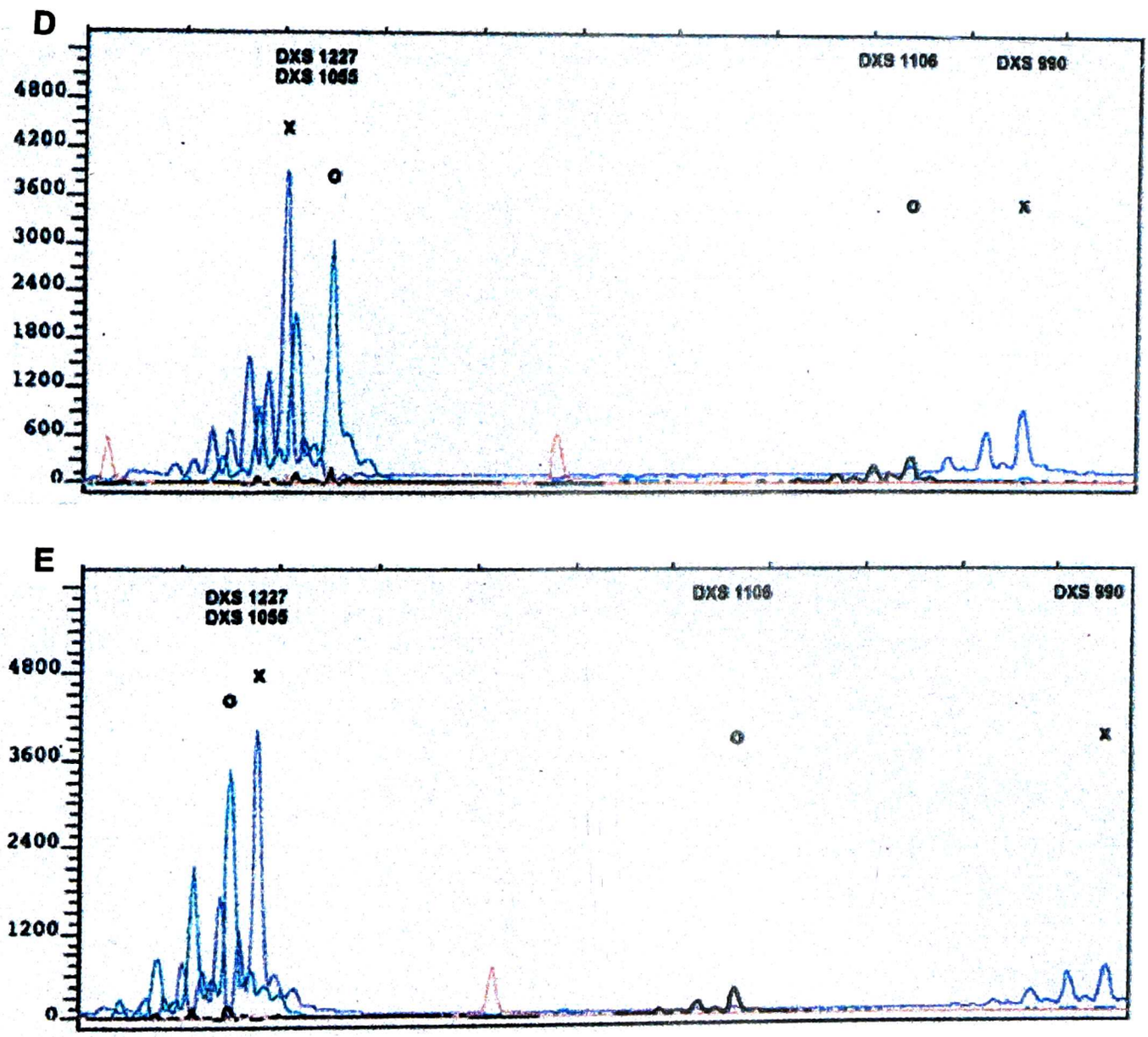

Figure 2. Genotypes generated by aRACE2 PCRs using aDNA extracts derived from five individuals recovered from the cemetery at al-Buhais. Alleles refer to the X-chromosomal STRs DXS1227 (blue, indicated by a cross), DXS1055 (green, open circles), DXS1106 (black, open circles), and DXS990 (blue, crosses). Markers used in this study are visualized in stack display. Panel A, individual alBCS; panel B, alBCR; panel C, alBCU; panel D, alBXB; panel E, alBAB. Note that an increase in allele size correlates with a decrease in peak height, thus indicating degraded template molecules.

Table 2. Amplification efficiencies obtained for five individuals from the Stone age burial ground at al-Buhais 18 using different combinations of aRACE1 primers

Sample	aRACE1 primers		aRACE2*	Genotype	aRACE1 primers		aRACE2*	Genotype
	DXS1227 (FAM) 76-96 bp				DXS1106 (HEX) 110-124 bp			
alBCS/m	A/AP2*	B/AP2	1 (out of 3)	87-87	A/AP2	B/AP2*	1 (out of 4)	114-114
alBCR/f	A/AP2*	B/AP2	1 (out of 2)	85-87	A/AP2	B/AP2*	1 (out of 4)	118-120
alBCU/f	A/AP2	B/AP2*	1 (out of 3)	81-87	A/AP2	B/AP2*	1 (out of 4)	112-118
alBXB/m	A/AP2*	B/AP2	1 (out of 3)	85-85	A/AP2*	B/AP2	2 (out of 4)	118-118
alBAB/?	A/AP2	B/AP2*	2 (out of 3)	87-87	A/AP2*	B/AP2	1 (out of 5)	112-112
	DXS1055 (TET) 77-93 bp				DXS990 (FAM) 122-130 bp			
alBCS/m	A/AP2*	B/AP2	3 (out of 3)	87-87	A/AP2	B/AP2*	1 (out of 5)	124-124
alBCR/f	A/AP2*	B/AP2	1 (out of 3)	83-87	A/AP2*	B/AP2	1 (out of 5)	124-130
alBCU/f	A/AP2*	B/AP2	1 (out of 3)	85-85	A/AP2	B/AP2*	1 (out of 5)	124-128
alBXB/m	A/AP2*	B/AP2	2 (out of 2)	87-87	A/AP2*	B/AP2	1 (out of 5)	124-124
alBAB/?	A/AP2*	B/AP2	1 (out of 4)	85-85	A/AP2*	B/AP2	3 (out of 8)	132-132

m = male, f = female, ? = unknown; A = labelled SP1 primer, B = unlabelled SP2 primer of the respective X-chromosomal STS tested; * the successful primer pair in aRACE2. The number of positive assays in relation to the total number of aRACE2 amplifications performed is indicated (column aRACE2).

printing using the STR loci DXS1227, DXS1055, DXS1106, and DXS990 was confirmed by 3% agarose gel electrophoresis. Table 2 summarizes results about aRACE1 primer combinations that were informative in aRACE2 PCRs. Alleles were identified using an automated DNA sequencer and specific detection software for the fluorochromes used (Figure 2). Interestingly, we did not observe any differences between the use of a fluorescence-labelled or an unlabelled primer, or various dyes (i.e. FAM, HEX, TET) used for labelling (Table 2).

Analysing ancient human DNA, however, always bears the risk of having amplified trace amounts of contaminating modern nucleic acids of human origin. Therefore, negative (i.e. ddH_2O) and blank extraction controls were run parallel to each extraction, and in addition to every nuclear-specific PCR assay we carried out positive controls running (i) in the same thermal cycler together with the aDNA amplifications, and (ii) separately from the aDNA amplifications in a second PCR cycler to test for cryptic "carry-over" effects (COOPER 1992). Moreover, as an alternative authentication, we tested the aRACE procedure on aDNA isolated from prehistoric pig and sheep, and obtained PCR products with the expected sizes (data not shown). In summary, taking into account the control results and the failure in obtaining larger PCR products, we conclude that the aRACE-generated amplicons mirror DNA segments that are endogenous to the respective individual.

Discussion

When extracting nucleic acids from prehistoric hard or soft tissues, the isolated DNA is in a more or less degraded state. To overcome the obstacles associated with severely degraded DNA, we needed to optimize the tools available for aDNA investigation (i.e. quality improvement of aDNA). It has been repeatedly shown that the amplification of high copy number templates (e.g. mitochondrial DNA, ribosomal DNA, chloroplast DNA) is feasible from non-modified nucleic acids. In contrast, the amplification of single copy nuclear DNA loci is a rare exception. aRACE was developed for amplification of nuclear aDNA in general, but especially for samples with D/L_{asp} contents far greater than the normal threshold of $\sim 80 \times 10^{-3}$.

Prior to aRACE the aDNA was repaired to produce blunt-ended duplex strands containing the target segment. In test series lacking the repair reaction (RR) we frequently obtained by-products (i.e. non-specific amplicons), possibly generated by PCR jumping, and generally a high background was noticed in PCRs. By-products of artificial origin were also generated using aRACE, but these fell outside the expected size range for the amplification products.

The ligation of adaptors to RR-modified aDNA leads to the creation of a population with the same terminal structure. This population is, therefore, essentially a library of uncloned aDNA fragments from which one may amplify many different targets using different sets of sequence-specific primers. Included within the primer binding sites for the AP1 and AP2 primers are restriction sites for the endonucleases *Not*I, *Srf*I, and *Xma*I. In principle, cloning of aDNA into plasmid or phage vectors is therefore possible, and consequently offers the means to immortalize vintage, prehistoric, ancient, and fossil genetic material. However, it has to be stressed that the step of adaptor ligation is crucial in aRACE. In a number of samples we noticed severe degradation of adaptor molecules and frequently alterations in the base order of the adaptor sequence. In other cases the ligation of adaptors to the aDNA failed, probably due to inhibitory agents present in the tissues. Therefore, it appears that the proper concentration of ligase enzyme has to be titrated individually, and that interfering PCR inhibitors co-purified with vintage nucleic acids should be completely removed.

FOO et al. (1992) developed a method that utilizes an alternative technique (generalized PCR; GPCR), but this has the aim of amplifying the modified DNA *in toto* by using a single primer complementary to the adaptor. Using this method, however, we obtained inconsistent results when amplifying a specific nuclear segment from this pool of randomly amplified DNA; and since FOO et al. (1992) targeted high copy number mitochondrial DNA for amplification, GPCR would appear to be much less sensitive than aRACE. In addition, we agree with the authors when they postulate that this technique enhances the possibility for jumping PCR (PÄÄBO et al. 1990, LAWLOR et al. 1991). In contrast, the Clontech adaptor provides a well tried and tested technology which has a number of features that fa-

vour the success of specific amplification by PCR. The specificity of aRACE is greatly enhanced by the absence of an AP1/2 binding site on the adaptor-ligated DNA. This site is created on the aDNA of interest by extension from the sequence-specific primer during the first cycles of aRACE. The amino group on the adaptor blocks extension of the 3`-end of the adaptor-ligated aDNA, and thus prevents formation of an AP1/2 binding site on the general population of aDNAs. This helps eliminating non-specific amplification among the population of aDNA fragments. Further, we could note that the aRACE1 reaction is favoured by the use of the AP2 primer. The AP1 primer was less successful, which was generally noted as a lower amplification efficiency in corresponding aRACE2 reactions, and may depend on the short sequence that partly matches the adaptor sequence in the early PCR cycles and/or on degradation/inhibitory effects as outlined above. Further improvement of aRACE success may be expected (i.e. amplification of amplicons >130 bp; general increase in the number of positive assays), when SP primers are designed that better match the annealing temperature of the respective AP1 or AP2 oligonucleotide (i.e. 64-69°C).

In this study, PCR products comprising di- and polymorphic single copy loci of human chromosomes X and Y were generated from 6 individuals. Whereas the anthropologically determined sex of the four individuals alBCS, alBCR, alBCU, and alBXB was indirectly confirmed by the presence of hemizygous (i.e. presumably male) or homo-/heterozygous (i.e. female) X-chromosomal genotypes, the individual alBAB gave an unclear typing using methods of anthropology and archaeology. For alBAB the X-chromosomal markers gave, in the 4 STR systems tested, allelic constellations of homozygosity. This strongly indicates that alBAB is of male origin, displaying hemizygosity of the markers, but it is not unequivocal evidence. To further clarify the sex of alBAB, an aRACE-mediated sex test similar to that performed on the Neresheim hominid is currently being employed.

Further, it is noteworthy that the amplification of DXS990 from alBAB is probably based on a stutter effect (SCHULTES et al. 1999) possibly due to repeated amplifications or due to the incorporation of additional bases during RR. This phenomenon provides evidence for the presence of severely degraded aDNA. On the other hand, hemizygosity 132 (or homozygosity 132-132, if female) exceeds the known maximum allele size of Caucasian populations by 2 bp, and might also be explained by either a novel allele not present within contemporary populations, or by an allele that has not been identified yet.

Apparently, aRACE has limitations regarding the maximum length of amplicons (~130 bp), and relies upon the fragmented state of ancient nucleic acids normally isolated from prehistoric bones. In other test series we attempted the amplification of the STR loci HumVWA, D17S799, and D18S478, which generate larger alleles with sizes between 134 bp and 253 bp. As with the initial experiments using standard techniques for the extraction and amplification of aDNA, we were unable to reproducibly amplify the correct PCR products with aRACE.

A single exception was a faint HumVWA-specific amplicon derived from individual alBAB (data not shown). In order to validate this result we have to further optimize the aRACE reaction.

Considering the problems associated with the amplification of larger alleles, it thus appears that a general restriction to amplicons smaller than 130 bp must be assumed when specimens with D/L_{asp} contents far greater than 80×10^{-3} are analysed by PCR. Consequently, our data show that even samples whose contents of D/L_{asp} are notably high may at least serve as informative templates using PCR systems that generate amplicon sizes of up to 130 bp.

Conclusions

The current high failure rate of palaeogenetic analyses calls for the development of novel techniques that might expand the scope of feasible studies. If aRACE proves successful in future studies, it may help in analysing traces of DNA purified out of ancient and maybe fossil specimens. Although the results presented here appear promising, it cannot be denied that the procedure struggles with a number of problems already recognized by others. For example, as with other methods, contamination cannot be monitored reliably. Thus, inconsistent results are expected when pool amplifications as described in COLLINS et al. (2000) will be performed on aRACE-modified aDNA. Inconsistency in general, the generation of artificial alleles, and results that were falsified by cryptic contamination of any source have also been noticed when STRs as well as mitochondrial segments were targeted applying contemporary nucleic acids as well as bones and teeth of up to 5,000 years of age (RAMOS et al. 1995, KOLMAN,TUROSS 2000, SOBRIDO et al. 2000). Since each microsatellite, even if modern DNA is used as template, displays certain patterns of artifact formation which must be recognized to avoid false-positive diagnoses (SOBRIDO et al. 2000), their usefulness appears to be strictly limited in aDNA research. Moreover, ancient (i.e. authentic) genotypes based on dinucleotide markers have been shown to be difficult to postulate. Since repeat sequences of different length interact with the partially degraded repeats of the amplified loci during thermal cycling, an even higher degree of artifact formation is expected. RAMOS and colleagues (1995) have therefore questioned if STR markers or single copy loci in general are reliable enough for genotyping ancient hard tissue samples.

However, the aRACE procedure, in principle, is a major leap towards the creation of an optimal – and yet not available – methodological improvement. Since such an improvement still awaits invention, there is a need for further technical innovations that will hopefully have a favourable effect on future palaeogenetics.

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