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An improved PCR method for endogenous DNA retrieval in contaminated Neandertal samples based on the use of blocking primers

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ABSTRACT

Neandertal skeletal remains are usually contaminated with modern human DNA derived from handling and washing of the specimens during excavation. Despite the fact that the distinct Neandertal haplotypes allow the design of specific primer pairs, for instance in most of the mitochondrial DNA (mtDNA) hypervariable region 1 (HVR1), the human contaminants can often outnumber the endogenous DNA, thus preventing a successful retrieval of Neandertal sequences. We have developed a novel PCR method, based on the use of blocking primers that preferentially bind to modern human contaminant DNA and block their amplification, and greatly improve the efficiency of Neandertal DNA retrieval. We tested the method in four El Sidrón Neandertal samples (two teeth and two bone fragments) with different contamination levels and taphonomic conditions, and we have been able to significantly increase the Neandertal yield from figures around 25.23% (5–69.6%) up to 90.18% (75.3–100%).

1. Introduction

Most ancient remains are to some extent contaminated with modern human DNA, as evidenced by the retrieval of human sequences from ancient cave bear and dog samples (Hofreiter et al., 2001; Malmström et al., 2005). At least some of these contaminants derive from handling and washing of the remains by the excavators (Sampietro et al., 2006; Melchior et al., 2008; Fortea et al., 2008). This complicates the possibility of working with ancient humans, since contaminants and endogenous sequences are often indistinguishable (Caramelli et al., 2003). Over the last decade, the polymerase chain reaction (PCR) has been used on DNA extracts from Neandertal specimens, to recover multiple mitochondrial DNA (mtDNA) hypervariable region 1 (HVR1) sequences (Krings et al., 1997, 2000; Ovchinnikov et al., 2000; Schmitz et al., 2002; Serre et al., 2004; Lalueza-Fox et al., 2005; Beauval et al., 2005; Lalueza-Fox et al., 2006; Orlando et al., 2006; Caramelli et al., 2006; Krause et al., 2007). A downside of conventional PCR, however, is that it can produce highly biased results, due to the potential co-amplification of contaminant sequences. In the specific case of Neandertals, one solution to this problem is through the design of primers that theoretically should preferentially target Neandertal, over modern human, DNA – for example by placing the 3′ end of the primer over Neandertal-specific nucleotide substitutions. However, even with the use of such highly specific Neandertal primers, studies have demonstrated that the resulting PCR products mainly consist of human contaminant sequences. For instance, the primer pair 16,230–16,262 (numbered according to CRS, the human reference sequence) that matches specific Neandertal substitutions in the L and H primers, including an adenine insertion between nt 16,263 and 16,264, has produced 95% contaminant sequences in El Sidrón 441 (Lalueza-Fox et al., 2005) and 95.5% in Vindija 77 (Serre et al., 2004). This is not surprising, because in some samples the amount of endogenous DNA is very low, and also because of the preferential amplification of the recent contaminants relative to the degraded original DNA. In addition, some sections of the HVR1 have fewer substitutions between Neandertal and human, thus making it difficult to place highly specific primers. For instance, from 16,022 to 16,122, most of Neandertals have only between one and three substitutions (in nt positions 16,037G; 16,078G and in some cases 16,093C). In a fragment of similar length (16,200–16,300), most Neandertals carry eleven substitutions (16,209C; 16,223T; 16,230G; 16,234T; 16,244A; 16,256A; 16,258G; 16,262T; 16,263bA; 16,278T

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Table 1
Primers designed for this study, numbered by the CRS (human reference sequence). Block: blocking primer; Ne: Neandertal-specific, L: Light strand, H: Heavy strand. Mismatches are underlined.

BlockF1 L16234
GTACAGCAATCAACCCTCAACTATCAC
BlockR1 H16256
GTTTGTTGGTATCCTAGTGGGTGAGGGGTGG
Ne1 L16230
GCACAGCAATCAACCTTCAACTG
Ne1 H16262
GGTTTGTTGATATCCTAGTGGGTGTAA
BlockF2 L16262
ATCACACATCAACTGCAACTCCAAAGCCACCCC
BlockR2 H16299
TAAATGGCTTTATGTACTATGTACTGTT
Ne2 L16244
CAACCTTCAACTGTCATACATCAACTA
Ne2 H16301
TAAATGACTTTATGTGCTATGTACTG

and 16,299G). This explains why in some specimens such as Scladina (Orlando et al., 2006) or Teshik Tash (Krause et al., 2007) it has been impossible to cover the first section of the HVR1, and also why in poorly preserved samples such as Vindija 77, La Chapelle-aux-Saints, Engis 2, El Sidrón 441 and Rochers de Villeneuve (Serre et al., 2004; Lalueza-Fox et al., 2005; Beauval et al., 2005), only the highly variable segments have been retrieved (yielding short sequences between 31 bp and 48 bp). It must be emphasized, however, that these samples contain authentic Neandertal DNA and that only our inability to retrieve it from the overwhelming contaminant background prevents us from having more genetic data from these extinct humans. The new ultrasequencing technologies, that can produce millions of sequences from ancient extracts, have been able to generate complete mitochondrial genomes from those exceptionally well-preserved samples (Green et al., 2008; Briggs et al., 2009). However, this approach is extremely inefficient in highly degraded and highly contaminated samples.

We have developed an alternate PCR-based method, based around the incorporation of so-called 'blocking primers' (Vestheim and Jarman, 2008) that are designed to preferentially bind to contaminant sequences, and subsequently block their amplification during the PCR. The method could easily be transferred to any degraded specimen, in which the endogenous sequence is known to be different from the potential contaminants.

2. Materials and methods

2.1. Neandertal samples

We have selected four El Sidrón Neandertal specimens that have been excavated between 2004 and 2006. El Sidrón is a karstic system in Asturias (North of Spain), where more than 1500 Neandertal remains belonging to nine individuals, have been recovered to date (Rosas et al., 2006). Although currently an excavation protocol that is designed to minimise human contamination of the samples is implemented at the site (Fortea et al., 2008), at least 140 samples were recovered before this in 1994, when the site was discovered, and are likely contaminated through unprotected handling and washing of the samples. These samples therefore offer a range of different preservation and contamination levels for study, mimicking the various scenarios found at many other Neandertal sites. The samples analysed here are: dentine root fragments from an adult incisor (SD 441) (Lalueza-Fox et al., 2005), an adult tooth (SD 1161) and two adult male femur fragments (SD 1253 and SD 1351c) (Krause et al., 2007; Lalueza-Fox et al., 2008, 2009).

Table 2
Ratio of endogenous Neandertal sequences versus human contaminant sequences with and without blocking primers.

Samples	Without blocking primers Neandertal/Human	% Endogenous DNA	With blocking primers Neandertal/human	% Endogenous DNA
441	4/80	5%	67/89	75.3%
1161	16/23	69.6%	23/23	100%
1253	8/50	16%	44/46	95.7%
1351c	10/97	10.3%	26/29	89.7%

2.2. DNA extraction and amplification

The bone samples were extracted following procedures previously described (Lalueza-Fox et al., 2005, 2006). Ten milliliters of EDTA (pH: 8; 0.5 M) were added to the tooth/bone powder overnight to remove mineral salts. After centrifugation, the supernatant was incubated overnight at 50 °C in a lysis solution containing 1 ml SDS 5%, 0.5 ml TRIS 1 M and 5 mg/ml of proteinase K. The sample was subsequently extracted with phenol, phenol–chloroform and chloroform–isoamyl alcohol and concentrated with centricons-30 columns (Millipore) to a final volume of 100 μl.

To ascertain the efficiency of the blocking primer approach, two primer sets that target two Neandertal HVR1 mtDNA overlaping fragments, were designed (Table 1). The two primer pairs yield amplicons of different length, and thus, because of this, are expected to work with different efficiency (Table 1). The first, shorter, amplicon (16,230–16,262) produces a 70 bp product (including primers) and the primers contain 3 and 4 mismatches relative to the human reference sequence in the L and H primers, respectively. The second primer pair (16,244–16,301) amplifies a longer product of 100 bp (including primers) and the primers bind with less specificity (3 and 2 mismatches in the L and H primers, respectively). Each primer set consists of a Neandertal-specific primer pair plus a human-specific blocking primer pair (Table 1). The blocking primers have been modified at the 3′ end with a C3 spacer to prevent the TaqDNA polymerase from extending it once they are annealed to the targeted DNA. The Neandertal primers are regular PCR primers that anneal and extend normally under the appropriate PCR conditions. It is thus expected that the blocking primers will bind to the human contaminants and prevent their amplification, and that the PCR product will be mainly Neandertal, even in heavily contaminated samples.

The contamination level of many of the El Sidrón samples was, *a priori*, known to be low, due to both the preservation condition of the material and the implemented anti-contamination procedures. For instance, the amplicon spanning np 16,230–16,262 yielded no contaminant sequences using standard primers in several bone samples (unpublished results). As such we focussed one of the assays (using primer pair 16,230–16,262) on two samples (SD 441 and SD 1161) (Lalueza-Fox et al., 2005), that were excavated prior to the adoption of the anti-contamination protocol. For the second primer pair (16,244–16,301), we used two well-preserved bone samples (SD 1253 and SD 1351c), with low contamination levels, but with highly fragmented template DNA.

A two-step PCR protocol was used, modified from a previously published protocol (Krause et al., 2006). Both steps included 2 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1X AmpliTaq Gold buffer, 2.5 mM $MgCl_2$ and 500 μM for each dNTP. In the first multiplex step, 150 nM of each primer pair (both standard and blocking primers) were included, in a final reaction volume of 20 μl. Primary amplification consisted of a 10 min activation step at 94 °C, followed by 27 cycles at 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 20 s. Five microlitre of a 1 to 10 dilution of the primary amplification product were used as a template for the second PCR.